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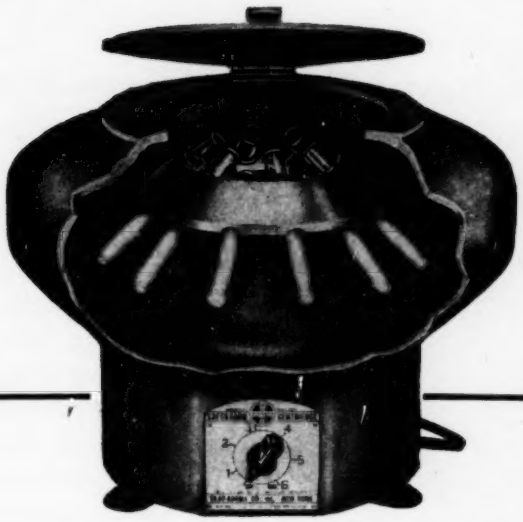

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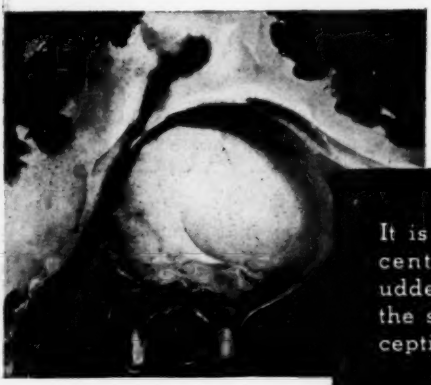

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Superovulation and Ovum Transfer in Cattle

RAYMOND E. UMBAUGH

San Antonio, Texas

IN THE FIELD of dairy and beef production, it is recognized that genetic characteristics determine the quality and economic efficiency of cattle. Artificial insemination has offered a means for the widespread distribution of desirable genes via the sperm. Similar genic selection through high quality females has, however, been limited, since a single cow will produce 1 calf per year, and the average number of offspring per female will seldom exceed 5, in a lifetime. It was felt that a marked increase in the production of offspring from desirable cows might be effected by superovulation, with transfer of the ova produced to less desirable recipients, who might act as host-mothers to the developing embryos. In mammals, this has been accomplished in the rabbit.[1,2,3,4] Furthermore, superovulation of the cow has been accomplished in a number of instances.[5,6,7]

Accordingly, the studies described in this paper were launched several years ago under the aegis of Mr. Tom Slick, scientific adviser to the Foundation of Applied Research. Various methods for the superovulation of cattle were tested, with the objective of being able to obtain large numbers of viable, transferable cow ova. At the same time, various techniques were devised for the transfer of such ova to recipient cows. Two major possibilities were explored: (1) the collection, fertilization, and transfer of ovarian ova, and (2) the collection and transfer of fertilized tubal and uterine ova. The first to be described are the experiments with ovarian ova and then the experiments with tubal and uterine ova. Certain collateral observations will conclude this discussion.

From the Foundation of Applied Research, San Antonio, Texas.

I wish to acknowledge my gratitude to Phyllis Umbaugh for valuable assistance, to Mr. Tom Slick and Dr. Gregory Pincus for advice, and to Dr. Wilbur Maker, who acted in the capacity of veterinarian for one and one-half years of the experiment.

*This term, as used, refers to unfertilized eggs or to zygotes in early cleavage stages.

I. METHODS OF COLLECTION AND TRANSFER OF COW OVARIAN OVA

Incisions Employed.—For the collection of ova* from exposed follicles, a ventral or flank incision was employed. For the ventral incision, the animal was completely anesthetized with chloral hydrate, rolled over on her back with the rump raised about 10 in. off the operating platform, in order to force the stomach and intestines to fall foward. The flank incision was made after the animal was prepared with an intravenous injection of chloral hydrate sufficient to cause drowsiness. Local anesthesia of the incision site was accomplished by injection with 50 cc. of a 2 per cent procaine solution. The animal's front feet were lowered 12 in. into a pit, so that the stomachs and intestines fell forward away from the reproductive organs and also from the incision. Usual aseptic precautions were taken at the incision sites.

Follicle Stimulation.—In order to effect the production of a large number of ripe ova, various methods of follicle stimulation were employed. The routine method finally evolved consisted of the implantation, subcutaneously into the neck region, of wax pellets containing 1,500 rat units (r. u.) of a sheep pituitary preparation. Except as noted hereinafter, ovarian corpora lutea were removed by palpation at the time of implantation. Four days after pellet implantation, 1,000 r. u. of the same extract dissolved in sterile physiologic saline were injected intravenously.** The intravenous injection induced a preovulatory follicle swelling that facilitated ovum withdrawal.

Collection of Ovarian Ova.—In order to take advantage of the follicular swelling preceding ovulation, ovarian ova were collected at twenty-six hours after the intravenous injection. At this time, no ovulation had yet occurred. After making the incision, the ovary was brought into the field by grasping the

**The sheep pituitary extract was G. D. Searle and Company's gonadophysin. We are indebted to Dr. A. L. Raymond, research director of the Searle Company, for the preparation of the pellets.

broad ligament and holding it close to the incision site. Aspiration of the exposed follicles was accomplished with a capillary pipette, the capillary portion of which was approximately 10 cm. long and the bore about 1¾ mm. The pipette tip was not fire polished, so that a cutting edge for follicle wall penetration was assured. The pipette tip was inserted just below the follicle wall surface, and the clear fluid withdrawn until the first spot of blood appeared. Deep penetration into the follicle was found inadvisable, since a viscous, bloody fluid, difficult to handle, was obtained. The aspirated fluid was discharged into a watch glass and observed under a dissecting microscope (cf. 2). Attempts were made to excise whole follicles prior to ovulation, and to obtain the ova therefrom *in vitro*. While ova could be taken from such follicles, the method proved tedious and caused such profuse hemorrhage from the ovaries as to make the method unsatisfactory. Similarly, attempts were made to collect ova from spontaneously rupturing follicles during ovulation. Most satisfactory was a glass container in which the ovary might be held and observed during the ovulation period. Since the ovulations are accompanied by greater or lesser amounts of hemorrhage from the follicle, the resultant fluid is difficult to use—ova could not be seen in the somewhat clotted, viscous exudate often filled with cellular débris.

Observations on Ovulation in Superovulating Cows.—In order to collect ripe, presumably fertile ovarian ova, and to determine the sequence of events following ovulation in animals stimulated to superovulation, the relation of ovulation time to the time of the intravenous gonadotrophin injection was determined. The pertinent data are collected in table 1. The data of table 1 are taken to demonstrate: (a) That superovulation will not occur unless the intravenous gonadotrophin injection is made (compare experiments 3, 11, and 17 with all the others); (b) that intravenous injection, as early as two days (experiments 7, 9, and 10) and as late as nine days (experiment 20) after pellet implantation, will induce superovulation; (c) that superovulation may be induced repeatedly in the same cow (experiments 4, 5, 6, 9, and 10); (d) that the three intravenous dosages used caused superovulation with about 21 ova per cow released with 500 r. u., about 38 with 1,000 r. u., and 29 with 1,500 r. u., but with considerable overlapping; and (e) that all pellet dosages used were compatible with superovulation, again with considerable overlapping in effect (e.g., dosages 1,000 to 1,750 r. u. yielded about 23 ova per cow, 2,000 to 3,000 r. u. yielded about 37 ova per cow).

Direct observation of the ovaries of 5 cows (experiments 8, 12, 18, 23, and 24) demonstrated a latent period of approximately twenty-four hours between the intravenous injection and the first appearance of ovulation. The liberation of ova may continue for at least four hours thereafter, and perhaps even for some days (*see* table 6). Since, during a four- to five-hour period, 18 to 20 ovulations were observed (experiments 12, 18, and 24), it is judged that a good crop of ova are liberated within a brief time after a fairly definite latent period.

The sequence of events at each ovulation is fairly uniform, and may be described as follows: First, the follicular wall is stretched and thinned to a point where slight membrane rupture permits the oozing of a serous follicular fluid which contains the ovum. Immediately thereafter, a viscous mucoid substance is expelled in the form of a stringy mass. This is followed by the appearance of a blood clot at the rupture point.

On the basis of the foregoing observations, it was decided to collect ovarian ova at about twenty-six hours after the intravenous injection, using the gonadotrophin regime described.

Collection and Transfer of Ovarian Ova.—In table 2 are presented data on the mean number of ova obtained from 13 cows and 34 heifers. It will be seen that, approximately, 6 ova per animal were obtained, whereas, judging by the rupture points in ovulated animals, an average of over 20 ova per cow should have been obtained (tables 1, 6, 7, and 10). Actually, approximately 20 follicles per cow were aspirated in this series. Two causes for failure to obtain the expected number of ova may be advanced: (a) failure to remove the ova because of improper pipette penetration and (b) the obscuring of the ova in the watch glass by overlying débris and blood clots.

Table 3 summarizes the data on the transfer of ovarian ova to the fallopian tube of recipient cows. None of the latter were subject to gonadotrophin, but were taken at the indicated times after the observation of a spontaneous, natural estrus. In a number of experiments, the ova were mixed in a watch glass with samples of bull semen picked up in a transfer pipette and transferred to the fallopian tubes of the recipient cow at one (experiment 2), two (experiment 3), and three (experiment 4) days after heat. Nine cows were artificially inseminated nineteen hours before the ova were deposited into the fallopian tubes (experiment 1) and, in 4 recipients, semen was first deposited in the fallopian tubes, and then, a few minutes later, the ova were transferred (experiments 5 and 6). None of these recipient cows became pregnant. The use of ovarian ova by these techniques, therefore, does not seem to hold promise as a method of egg transfer. Fertilization of freshly ovulated rabbit ova *in vitro* by semen

TABLE I—Superovulation by the Use of Sheep Pituitary Pellets

Experiment (No.)	Cow (No.)	Implanted	Intravenous after implantation Days	Intravenous after implantation Amounts	Days after intravenous slaughtered	Palpated No. days after intravenous	Points (No.)	Remarks
1	1	1,500 r.u.	4	500 r.u.		4	16	
2	2	1,000 r.u.	3	1,000 r.u.		4	11	
3	4	1,500 r.u.		None				Did not ovulate
4	5	1,500 r.u.	3	1,500 r.u.		4	9	
5	5	2,000 r.u.	4	1,000 r.u.		5	33	Abdominal incision
6	5	2,950 r.u.	4	1,000 r.u.				Abdominal incision
7	7	3,000 r.u.	2	1,000 r.u.		5	43	
8	8	3,000 r.u.	3	1,000 r.u.		3	70	
9	10	1,500 r.u.	2	1,500 r.u.		8	20	
10	10	1,500 r.u.	2	500 r.u.		6	12	
11	11	1,500 r.u.		None				Did not ovulate
12	13	2,500 r.u.	4	1,500 r.u.			21	By observation
13	16	1,500 r.u.	4	1,000 r.u.		10	25	
14	19	2,000 r.u.	5	1,500 r.u.	10		76	
15	23	2,000 r.u.	3	500 r.u.		2	16	
16	26	2,000 r.u.	5	500 r.u.		6	47	
17	28	2,000 r.u.		None				Did not ovulate
18	28	2,000 r.u.	3	1,500 r.u.			18	By observation
19	29	1,500 r.u.	3	1,000 r.u.			28	Abdominal incision
20	30	2,000 r.u.	9	500 r.u.	5		13	
21	32	1,500 r.u.	4	1,000 r.u.	9		62	
22	33	1,500 r.u.	3	1,000 r.u.			22	Abdominal incision
23	35	2,000 r.u.	4	1,000 r.u.				
24	36	1,750 r.u.	3	1,000 r.u.			20	By observation

Experiment 5.—Pellets were implanted sixty days after the first implantation of pellets. Made abdominal incision on fifth day after the intravenous injection. Corpora lutea were counted on both ovaries and the right fallopian tube removed. Eight unfertilized ova were found after flushing the tube.

Experiment 6.—Pellets were implanted 128 days after the second implantation of pellets. Aspirated 23 follicles and found 23 ova. Ova were mixed with bull semen and put back in left fallopian tube. Cow was slaughtered two days later, and five unfertilized ova were flushed from the left tube.

Experiment 8.—Ovaries were exposed by an incision through the vagina twenty-eight hours after the intravenous injection, and it was observed that the ovaries had begun ovulating.

Experiment 12.—Twenty-seven hours after the intravenous injection, a flank abdominal incision was made so that ovulation could be observed. One follicle had ovulated at the time of making the incision and, by the end of four hours, 20 more follicles had ovulated. Two follicles were aspirated prior to ovulation and 2 ova were found.

Experiment 15.—The right ovary was removed twenty-four hours after the intravenous injection and ova aspirated out of the individual follicles. Sixteen follicles ovulated on the left ovary.

Experiment 18.—A flank abdominal incision was made twenty-four hours after the intravenous injection. The cow's front feet were lowered 12 in. so that ovulation could be observed with ease. Ovulation began twenty-eight hours after the intravenous injection and continued for five hours longer. Eighteen follicles ovulated.

Experiment 19.—Five days after the intravenous injection, removed the right fallopian tube (right ovary had 10 ovulation points). Flushed contents of tube and no ova were recovered. Removed the right uterine horn and flushed out 6 ova; 1 in four-cells, 1 in six-cells, and 4 unfertilized. The cow had been inseminated at time of the intravenous injection.

Experiment 20.—Flushed 2 ova from the uterus after slaughtering; 1 in two-cells and 1 in six-cells. The cow had been inseminated twenty-four hours after the intravenous injection.

Experiment 22.—Six days after the intravenous injection, the left uterine horn was removed. Four unfertilized ova were flushed from the removed uterine horn. This cow had been inseminated at the time of the intravenous injection.

Experiment 23.—Seventeen hours after the intravenous injection, a flank incision was made and both ovaries removed. There were 41 mature follicles on the left ovary and 34 on the right ovary. These follicles were aspirated and 20 ova were recovered.

Experiment 24.—Nineteen and one-half hours after the intravenous injection, a flank abdominal incision was made and the ovaries observed for thirteen hours. Twenty-seven and one-half hours after the intravenous injection, the ovaries began ovulating and continued to ovulate for four more hours.

is not possible, perhaps because of ovicidal seminal substances (Chang, unpublished data), so that experiments 2 and 6 may have failed to result in pregnancy for analogous reasons. We would have expected fertilization and development of the ova of experiment 1, however, since this involves merely an addition of exogenous ova to the oviducts, with presumably an adequate supply of sperm. Since, however, most of the recipients received a large number of ova, it is possible that resorption occurred of the type reported by Casida *et al.*[8] in superovulated sheep. John Hammond, Jr.,[9] has had evidence of failure to carry to term in superovulated cows carrying 4 or more embryos. Some of the cows in experiment 1 may have had undetected abortions in the field.

TABLE 2—Collection of Ova by Follicle Aspiration*

	No. animals	Ova collected (No.)	Ova per animal (Ave. No.)	Remarks
First collection				
Cows	13	78	6.0	
Heifers	34	219	6.4	
Second collection				
Cows	2	3	1.5	Adhesions on ovaries
Heifers	2	11	5.5	Adhesions on ovaries

*Follicles, not aspirated by the twenty-eighth hour after the intravenous injection, would usually begin ovulating.

Cow ova have been transferred to the fallopian tubes of rabbits after mixing the ova *in vitro* with bull semen (table 4, experiments 2, 3, and 5 to 8). The rabbits were sacrificed at various intervals thereafter, and the oviducts flushed with Ringer-Locke solution to recover the transferred ova. Of 35 such transferred ova, 23 were recovered and only 2 (experiment 6) gave any evidence of having been fertilized. Two rabbits were inseminated with bull semen twelve hours before ovum transfer (experiments 1 and 4), but only 3 of the 20 transferred ova were recovered with no evidence of development.

II. Methods of Collection and Transfer of Fertilized Cow Ova

In all the experiments of this group, artificial insemination of the gonadotrophin-treated cows was used. Deep insemination into the uterus was practiced at the time of the intravenous gonadotrophin injection and again twenty-two hours later.

Ova were taken from the fallopian tubes and uteri after slaughter, in a number of animals. Ordinarily, they were flushed out with cow serum. The time between slaughter and ovum recovery was one-half to three hours.

The same washing fluid was used in obtaining ova from living animals. Ova, transferred to the recipients, were collected from the live animal. For the collection of tubal ova, a small flank incision was made so that the ovaries, fallopian tubes, and uterus were in view. Care was taken not to damage or touch either the fallopian tubes or the ovaries, but the uterus was held in the hand and a small pointed catheter was forced through the uterine wall into the lumen. The catheter was run down to the uterine isthmus,[9] and the uterine horn grasped firmly back of the catheter to prevent fluid from running back toward the cervix and to allow sufficient pressure to build up to force the fluid into the fallopian tube. Care was taken to limit the amount of pressure used to avoid the chance of rupturing the uterine wall. A small glass container was held at the lowest point of the fimbriated end of the tube, and about ten drops of the issuing fluid collected. This amount of fluid appeared to contain all the ova present. A similar technique has been employed for the collection of human tubal ova[10] *in vivo*.

Ova were collected from the uterus by mak-

TABLE 3—Transfer of Ovarian Ova to Fallopian Tubes

Experiment (No.)*	No. cows used	Days before transfer heat observed (No.)	Ova transferred (No.)	Attempted fertilization method
1	9	1	98	Recipient bred 19 hours before transfer
2	4	1	19	Ova mixed with bull semen prior to transfer
3	4	2	14	Ova mixed with bull semen prior to transfer
4	10	3	25	Ova mixed with bull semen prior to transfer
5	2	1	21	Semen placed in ovarian end of tube prior to transfer
6	2	2	10	Semen placed in ovarian end of tube prior to transfer

*None of the above animals became pregnant.

ing a ventral incision, exposing the uterine horns, cannulating at the cervical and uterine horn ends, and flushing the fluid out through the cannulated uterine tip. Here, a larger amount of fluid was collected, enough to fill the bottom of a Petri dish. Attempts were made licles to a condition for superovulation. Using the regular ovulating intravenous dose did not cause ovulation. Furthermore, observation of the ovaries after slaughter (experiments 1 and 3), or by palpation, indicated no increase in follicles of ovulable size. Accordingly, in

TABLE 4—Cow Ova Transplantation in Rabbits

Rabbit (No.)	Intravenous injection before transfer (hr.)	Method of fertilizing	Cow ova transferred (No.)	Slaughtered after transfer (hr.)	Ova recovered and condition
1	19	Inseminated 12 hours before transfer	9	18	2—no cleavage
2	80	Mixed with bull semen	6	72	4—unfertilized
3	12	Mixed with bull semen	8	48	2—disintegrating
4	19	Inseminated 12 hours before transfer	11	46	1—in two cell disintegrating
5	6	Mixed with bull semen	6	26	4—unfertilized
6	16	Mixed with bull semen	4	20	2 in two cells, 2 disintegrating
7	13	Mixed with bull semen	9	25	5—unfertilized
8	27	Mixed with bull semen	6	24	4—unfertilized

with 2 cows (table 1, experiments 1 and 10) to withdraw ova via the cervix. One liter of physiologic saline was introduced into the uterus and approximately 300 cc. could be withdrawn. There were no ova in the withdrawn fluid, but 1 of the 2 cows (table 1, experiment 10) became pregnant, dropping twin calves at seven months. This cow had a positive agglutination blood test for brucellosis.

In transferring ova to the fallopian tubes, part of a uterine horn is pulled to the flank incision site, thus exposing the tubal fimbriae. The capillary of the transfer pipette is inserted about ¾ in. into the tube and the ova gently expelled. Care must be taken to pick the ova up in an amount of fluid that will not rise above the capillary, as they may become stuck to the sides of the pipette barrel. Ova were transferred to the uterus either through the flank incision with minimum puncture to the lumen, by the transfer pipette, or by insertion through the dilated cervix as in deep artificial insemination.

Attempted Superovulation by the Use of Pregnant Mare's Serum.—Three cows (table 5) received pregnant mare's serum* injections subcutaneously, in an attempt to stimulate follicles to a condition for superovulation. Using the regular ovulating intravenous dose did not cause ovulation. Furthermore, observation of the ovaries after slaughter (experiments 1 and 3), or by palpation, indicated no increase in follicles of ovulable size. Accordingly, in all subsequent studies, the priming doses of gonadotrophin were sheep pituitary extract.

Fate of Superovulated Ova in the Oviducts.—Forty-one cows, given pellet implants and the standard intravenous ovulating dose, were inseminated and slaughtered at intervals as indicated in table 6. Both the tubes and uteri were flushed for ova. Although an average of 23.4 ova per cow were ovulated as determined by the number of rupture points, only 10.9 (47 per cent) were recovered. Although most of the ova collected were in the fallopian tubes at the third day after the intravenous injection, some (6 per cent of those ovulated) were in the uteri and, by the fourth day, they tended to preponderate in the uterus. By the third day after the intravenous injection, one would expect, in the normal course of events, to have all the ova fertilized and started in cleavage, since most of the ova are ovulated by the thirty-first hour after the intravenous injection. Nonetheless, only 196 out of the 371 collected (53 per cent) cleaved. Similarly, of the 75 ova collected on days four to six, only 40 (again 53 per cent) were cleaved. The ova demonstrably fertilized, therefore, amounted to 236 or less than 25 per cent of the total number ovulated, and they averaged 5.8 per cow. Among the cleaved ova, any stage from two to about 16 cells were observed, often

TABLE 5—Attempted Superovulation by Pregnant Mare's Serum

Cow (No.)	Stimulation	Intravenous on fifth day	Slaughtered	Results
867	250 I.U. daily for four days	1,000 r.u. gonadophysin	5th day after intravenous	No ovulation, no stimulation
617	5,000 I.U. daily for three days	1,000 r.u. gonadophysin	Palpation	No ovulation, no stimulation
946	2,000 I.U. daily for four days	1,000 r.u. gonadophysin	5th day after intravenous	No ovulation, no stimulation

*We are indebted to Dr. E. C. Reifenstein, Jr., for supplies of Ayerst, McKenna, and Harrison's Antex.

TABLE 6—Position of Ova in Superovulated Cows on Varying Days of Slaughter After Intravenous Injection

Day of slaughter	No. cows	Ova ovulated (No.)	Average ovulated per cow	Collected per cow (No.)	(%)	Ova collected in tubes (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum	Ova collected in uterus (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum
3	33	763	23.1	11.2	48	324	42	139	185	3.4	47	6	36	11	6.2
4	5	95	19.0	6.4	33	9	9	1	8	8.4	23	24	10	13	5.4
5	1	48	48.0	16.0	33	0	0	0	0	0.0	16	33	16	0	0.0
6	2	56	28.0	13.5	48	0	0	0	0	0.0	27	48	8	19	5.4
	41	962	23.4	10.9		333		140	193	3.6	113		70	43	5.6

The animals in these experiments all ovulated one or more ova. Some follicles had ovulated only shortly before slaughtering.

TABLE 7—Ova Collected from Superovulated Heifers*

No. heifers	Hours after intravenous operation (No.)	Ova ovulated (No.)	Ova collected in tubes (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum	Ova collected in uterus (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum
8	46	194	64	33	62**	2	4.0	0	0	0	0	0
5	72	97	18	18	5	13	3.4	0	0	0	0	0
22	96	389	89	23	80	9	8.2	16	4	15	1	3

*Ventral incisions were made to make tubes and uterus more accessible. Animal was completely anesthetized.
**Nineteen of these ova were beginning to cleave.

TABLE 8—Varying Intravenous Injections of International Units of Pregnancy Urine Gonadotrophin

No. cows	Dosage of pellets	Intravenous of I.U. of P.U.*	Day slaughtered after intravenous	Ova ovulated (No.)	Ova collected in tubes (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum	Ova collected in uterus (No.)	(%)	Ova in one cell (No.)
14	2,000	2,000	3	11	3	27.0	3	0	0	0	0	0
7	2,000	10,000	3	37	2	5.4	0	2	2	0	0	0

*International units of pregnant urine.

TABLE 9—Subcutaneous Injections of Stilbestrol

Cow (No.)	Amt. of stilbestrol (mg.)	Days given after intravenous	Days slaughtered after intravenous	Ova ovulated (No.)	Ova collected in tubes (No.)	Ova collected in tubes (%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum
928	50	1	2	3	2	67	1	0	0
496	50	2	3	24	3	12	3	0	0
207	50	2	3	32	3	9	2	1	3
126	50	1	3	0	0	0	0	0	0
371	50	1	3	4	2	50	2	0	0

TABLE 10—Position of Ova in Superovulated Cows Given Varying Dosages of Progesterone at Varying Hours and Slaughtered at Varying Days After the Intravenous Injection

Day of slaughter	No. cows	Progesterone (mg.)	Hours after intravenous (No.)	Ova ovulated (No.)	Ova collected in tubes (No.)	Ova collected in tubes (%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum	Ova collected in uterus (No.)	Ova collected in uterus (%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum
3	2	50 50	24 49	45	23	51	20	3	2.0	8	18	5	3	2
3	3	25 25	23 46	31	18	58	8	10	4.1	0	0	0	0	0
3	1	100 100	23 46	12	7	58	2	5	4.2	0	0	0	0	0
4	1	75	24	31	13	45	0	13	4.3	7	23	6	1	3
4	2	25 25 25	24 48 72	31	0	0	0	0	0.0	10	34	10	0	0
4	2	50 50 50	24 48 72	43	1	2	1	0	0.0	3	7	3	0	0
4	1	100 100 100	24 48 72	20	4	20	4	0	0.0	1	5	1	0	0
6	1	25 25 25 25 25	24 48 72 96 120	4	0	0	0	0	0.0	1	25	1	0	
6	3	50 50 50 50 50	24 48 72 96 120	32	0	0	0	0	0.0	4	12	4	0	0

side by side in the same tube or uterus, although the mean blastomere number per ovum was 3.4 among the three-day-cleaved tubal ova and 8.4 among the four-day-cleaved ova. The cleaved uterine ova were, on the average, less advanced than the four-day-cleaved tubal ova.

Since slaughtering might in some way have affected the position and condition of the ova, somewhat comparable data were obtained on ova collected from the living cow. These are presented in table 7, which summarize the data on 35 superovulated heifers. The earliest ova were taken at forty-six hours after the intravenous injection. According to the number of rupture points, 680 ova, or 19.6 per cow, were ovulated; 187 (28 per cent) were collected and, of these, approximately 44, or less than 1.3 per cow, were fertilized. In this series, the fertilized tubal ova taken at forty-six hours appear to be at the beginning of cleavage, whereas, the four-day (96-hour)-cleaved ova average 8.2 blastomeres.

The number of ova, the number recoverable, and extent of fertilization appear, therefore, to be even less among these heifers than among the slaughtered cows. In a small series (5 cows), collection of tubal ova from anesthetized cows three days after the intravenous injection was conducted. An average of 20.6 rupture points per cow were observed; but only 2.6 per cow were flushed from the tubes, and, of these, only 1 per cow was cleaved. In both series, however, there appears to be a considerable loss of ovulated ova and a hindrance to fertilization. There is some indication, also, of somewhat early passage of some ova into the uterus (table 6, three-day postinjection data) and of poor development of ova after entry into the uterus. This is in contrast to the situation in the rabbit, where practically all superovulated tubal ova are fertilized and recoverable.[9]

To ascertain if normally ovulating cows might exhibit such inhibition of fertilization, the tubes of 7 cows inseminated at normal estrus were flushed via a flank incision. Ova were obtained from 4 of them and these were in the expected states: 1 just cleaving when removed a day after the conclusion of heat, 1 in four cells, and 2 in six cells when taken at two days after heat. This compares with the data of Hamilton[11] on normal cow ovum development.

Since some feature of this hormone treatment seemed to contribute to loss of ovulated ova and prevention of fertilization, a number of variations were attempted, in order to assure the uniform production of large numbers of fertilized ova.

Twenty-one cows, given a 2,000-r. u. pellet implant treatment, were injected intravenously with chorionic gonadotrophin of pregnancy urine* and inseminated. The data of these experiments are presented in table 8. Neither dose employed improved the yield of collectable or fertilized ova; in fact, the yield decreased considerably over comparable experiments using sheep pituitary gonadotrophin (cf. table 6).

The possibility of overproduction of either progesterone or of estrogen by the stimulated ovaries was considered as a possible cause of the loss of ova and fertilization inhibition. In tables 9 and 10 are presented data on the effects of administering estrogen (stilbestrol) and progesterone** to cows receiving pituitary gonadotrophin.

The stilbestrol treatment (table 9) was ineffective in increasing yield (10 of 63 ovulated ova recovered, of which only 1 was cleaved) and may even have inhibited ovulation in 1 case (cow 126).

The data on progesterone administration are presented in table 10. In these experiments, a total of 219 rupture points were observed, but again only 100 ova (46 per cent) were recovered and, of these, only a portion appeared to be fertilized. This is especially obvious in the ova collected from the uterus, for, out of 34 such ova, only 4 were cleaved. There appears, furthermore, to be no obvious relationship between the dosage of progesterone used and the fate of the ovulated ova. Thus, in animals slaughtered at three days, the 50-, 100-, and 200-mg. dosage animals gave, respectively, 58, 69, and 58 per cent recoveries of ovulated ova, and the per cent fertilized in the three groups was 32, 13, and 42, respectively. Similarly, the even lower recoveries at four days give no regular relation to progesterone dosage.

It was felt that the loss of ova and the inhibition of fertilization in cows superovulated by the foregoing methods might be ascribed to rapid overstimulation of the ovaries. Accordingly, a subcutaneous (priming) injection schedule of five days was instituted with the intravenous injection made on the sixth day. The results of varying the dosages of both the subcutaneous and intravenous injections are given by the data of table 11. The results indicate that a relatively low daily priming dose (50 to 100 r. u. per day) with a low intravenous ovulating dose (25 to 50 r. u.) allows for maximal recovery of ovulated ova. Thus, in the cows receiving 500 r. u. per day (table 11, experiments 6 and 7), no tubal ova were recovered on slaughter four days after the intravenous injection, and, in but 1 of the 2 cows, 4 unfertilized ova were obtained from the uterus, representing 4 out of a total of 46 ova

*Ayerst, McKenna, and Harrison's APL kindly contributed by Dr. E. C. Reifenstein, Jr.

**Kindly contributed by Dr. E. Schwenk of the Schering Co

TABLE 11—Superovulated by Subcutaneous Injections over Five-Day Period and Intravenous Injection on the Sixth Day

Experiment (No.)	No. cows	Daily dosage (r.u.)	Amt. intravenous given (r.u.)	Days after intravenous slaughtered	Ova ovulated (No.)	Ova collected in tubes (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum	Ova collected in uterus (No.)	(%)	Ova in one cell (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum
1	8	100	50	3	61	40	66.0	8	32	4.9	1	1.6	1	0	0.0
2	2	100	25	3	13	11	85.0	7	4	4.3	0	0.0	0	0	0.0
3	1	100	1,400	4	47	0	0.0	0	0	0.0	0	0.0	0	0	0.0
4	1	100	500	4	16	2	12.0	0	2	2.0	4	25.0	4	0	0.0
5	1	200	1,000	4	57	1	1.7	1	0	0.0	1	1.7	1	0	0.0
6	1	500	1,000	4	18	0	0.0	0	0	0.0	0	0.0	0	0	0.0
7	1	500	500	4	28	0	0.0	0	0	0.0	4	14.0	4	0	0.0
8	1	50	480	3	8	7	87.0	2	5	4.4	0	0.0	0	0	0.0
9	6	100	100	3	73	15	22.0	13	2	8.0	10	13.0	10	0	0.0
10	6	100	250	3	114	33	29.0	32	1	2.0	12	11.0	10	2	2.5
11	1	50	300	4	28	5	18.0	4	1	3.0	2	7.0	1	1	3.0
12	2	50	75	4	59	8	14.0	8	0	0.0	0	0.0	0	0	0.0
13	3	100	100	5	13	0	0.0	0	0	0.0	9	69.0	9	0	0.0
14	9	100	100	2	74	35	44.0	30	5	3.2	4	5.0	4	0	0.0
	43				627	157		105	52	4.6	47		44	3	2.7

TABLE 12—Superovulation by Subcutaneous Injection over Five-Day Period and Intravenous Injection on the Sixth Day

No. cows	Daily dosage (r.u.)	Amt. intravenous given (r.u.)	Days after intravenous operated*	Ova ovulated (No.)	Ova collected in tubes (No.)	(%)	Ova in one cell** (No.)	Ova in multiple cell (No.)	Average cell No. per cleaved ovum
7	125	125	3	78	12	15	9	3	2.3
4	150	150	2	66	29	44	29	0	0.0
9	300	300	2	97	22	23	22	0	0.0
7	150	300	2	158	56	35	54	2	2.0
2	250	300	3	19	7	37	7	0	0.0
29				418	126		121	5	2.2

*Ova collected by making flank incision and washing the fallopian tubes.
**Many of the one cell ova divided subsequently in culture indicating fertility.

ovulated. That an overlarge intravenous injection may also contribute to ovum loss and fertilization inhibition is indicated by the data of experiments 3 to 7, in which intravenous doses of 500 to 1,400 r. u. were employed; 166 ova were ovulated and only eight unfertilized ova recovered in tubes and uterus. In the 8 cows receiving somewhat lower intravenous dosages (experiments 8, 10, and 11), 59 of 150 ovulated ova were recovered, and only 17 were cleaved. The cows (experiment 13) on low dosage, slaughtered at the fifth day after the intravenous injections, had 69 per cent of ovulated ova, although unfertilized, in the uterus, and the cows (experiment 14) on the same dosage, slaughtered on the second day, ovulated 74 ova, of which 49 per cent were recovered, mostly in the tubes. In the remaining experiments (1, 2, and 12), in which the lowest priming and intravenous dosages were used, 133 ova were ovulated and 60 collected. Of these, 36 were cleaved, averaging 3 per cow.

Using similar injection schedules, ova were collected from the fallopian tubes of 29 living cows, flushing out the ova at the second and third days after the intravenous injection. The data are presented in table 12. Of the 418 ova ovulated, 126 (30 per cent) were collected. Most of these ova were in one cell, but culture of a number of them resulted in cleavage indicating the probability of fertilization. With this type of injection schedule, we have not determined the exact time of ovulation, so that fertilization time and cleavage rates are not obvious. From the data of table 11, it is clear that, by three to four days after the intravenous injection, cleavage was under way in some cows. In the one series slaughtered on the second day (experiment 14), 9 of 39 ova were in cleavage. The data on the slaughtered cows (table 11), indicate that the dosages employed in the experiments of table 12 were, if anything, too high to allow for maximal ovum recovery.

It is clear from the foregoing that full recovery of superovulated ova and maximal fertilization have not been attained, although low dosage experiments of table 11 are very suggestive. Even in the most productive of these experiments (1 and 2, table 11), the average return of cleaved ova was 3.6 per cow out of 7.4 per cow ovulated. It should be noted that the rate of travel into the uterus of ova ovulated by the five-day priming schedule is probably slower than in the pellet experiments (table 6), since most of the ova obtained at the third and fourth days after the intravenous injections were in the tubes, very few in the uteri. Also, the proportion demonstrably fertilized (*i. e.*, cleaved) were in the pellet implantation experiments, less than 25 per cent of the total ovulated, whereas, in the lowest dosage experiments on the five-day priming schedule (experiments 1 and 2, table 11), about 48 per cent of the ovulated were fertilized.

Results of the Transfers of Superovulated Ova.—Sixteen ova were collected (9 in one cell, 1 in two cells, 2 in three cells, 2 in four cells, and 2 in eight cells) and transferred to the fallopian tube of a Guernsey recipient that was in heat the previous day. This recipient was isolated and she was not inseminated. She missed the first heat period and rectal palpation, at thirty-five days, indicated a pregnancy. At fifty days, pregnancy in both horns was indicated. At one hundred days, both uterine horns were very large and definitely had embryos. At 112 days, she aborted. She was in the pasture and it was impossible to find the embryos.

The second cow had 17 ova transferred to her fallopian tube. These ova were in one cell. She had her own ovulated ovum flushed from her fallopian tube. The cow became pregnant, but aborted at sixty-five days.

Another cow had 1 four-cell ovum transferred to her fallopian tube. She had her own ovulated ovum flushed from her fallopion tube. At 104 days, she aborted 1 embryo. This cow was suspicious on the agglutination blood test for brucellosis. The four-cell ovum was collected from a normal ovulating cow that was in heat and artificially inseminated two days before making the transfer. The evidence that superovulated cows, carrying 4 or more embryos, fail to carry to term (cf. 9) may possibly explain the abortions in the 2 cows carrying several embryos.

SUMMARY

Methods for the collection of ripe ova from stimulated ovarian follicles are described. The implantation of wax pellets, containing about 1,500 r.u. of pituitary gonadotrophin followed, in three to four days, by an intravenous injection of gonadotrophin, leads to the ripening of large numbers of follicles to the ovulable condition. Such follicle stimulation appears to be best achieved by pellet implantation shortly after the removal of an active corpus luteum. Ova are collected from the swelling follicle apex just before ovulation, at twenty-six hours after the intravenous injection. Superovulation from such follicles occurs, the first follicle rupture beginning no sooner than twenty-four hours after the intravenous injection and subsequent ovulation continuing for several hours. Superovulation will not occur unless the intravenous gonadotrophin is given. Fertilization of ripe ovarian ova with semen *in vitro* and transfer to recipient cows were attempted, but no calves were obtained. Similarly, the transfer of ovarian ova to the fallopian tubes with

semen or to the fallopian tubes of inseminated cows gave no calves. Attempts to fertilize cow ovarian ova in the oviducts of rabbits resulted in, at best, a few cleaved ova.

Superovulation by the use of pregnant mare's serum was not accomplished, but pituitary gonadotrophin administration led to superovulation in several series of cattle. In cows superovulated by pituitary extract pellet implantation, followed by intravenous injection, an average of 23.4 ovulation points per cow was obtained. In a series of artificially inseminated animals, slaughtered at three to six days following the intravenous injection, 10.4 ova per cow were recovered from the oviducts. Of these 10.4 per cow, 5.8 per cow were fertilized, as indicated by cleavage stages. A number of these superovulated ova appeared to descend prematurely into the uterus from the fallopian tubes.

Methods for collecting superovulated ova from the fallopian tubes and uteri of living sperm-inseminated cows are described. Such collections, from a group of 35 heifers at two to four days after the intravenous injection, led to a recovery of 28 per cent of the ova ovulated. Cleaved ova in this series averaged 1.3 per heifer.

Attempts to increase the percentages of fertilized ova from superovulated, sperm-inseminated cows by (a) using chorionic gonadotrophin for the intravenous ovulating injection and (b) accompanying or following gonadotrophin administration with estrogen or progesterone were unsuccessful.

A five-day injection schedule of priming doses of pituitary gonadotrophin, followed on the sixth day by an intravenous ovulation injection, also accomplished superovulation, and such ova could be collected from the oviducts. A schedule of low dosage gonadotrophin injection by this method led to the recovery of a large portion of the superovulated ova and, of these, over half were fertilized.

The pregnancies resulting from the transfer of superovulated ova to recipient cows are described.

DISCUSSION

It was observed that cows having their corpora lutea removed hemorrhaged large amounts of blood, thus causing adhesions which could seriously affect the fertility of the cow. Also, many cows "deep" inseminated had slight-to-severe uterine infections from the deep inseminations.

A total of 750 cows was used in these experiments over a period of eight years.

The percentages of pregnancies resulting from the transfer of ova, in varying stages of development, to the tubes and uteri of recipients will follow in a subsequent paper.

References

[1]Heape, W.: Preliminary Note on the Transplantation and Growth of Mammalian Ova Within a Uterine Foster-Mother. Proc. Roy. Soc., London, *48*, (1890):457.

Heape, W.: Further Note on the Transplantation and Growth of Mammalian Ova Within a Uterine Foster-Mother. Proc. Roy. Soc., London, *62*, (1897):178.

[2]Pincus, G.: The Eggs of Mammals. The MacMillan Co., New York, 1936.

[3]Chang, M. C.: Probability of Normal Development After Transplantation of Fertilized Rabbit Ova Stored at Different Temperatures. Pro. Soc. Expti. Biol. and Med., *68*, (1948):680.

[4]Chang, M. C., and Pincus, G.: The Artificial Insemination of Rabbits and the Transplantation of Rabbit Eggs. (Motion picture). Federation Proc., *8*, (1949):23-24.

[5]Zawadowski, M. M.: Stimulation of Multi-Births in Farm Animals. Reserves of Animal Husbandry, Moscow, 1945.

[6]Hammond, J., Jr., and Hattacharya, P. J.: Control of Ovulation in the Cow. J. Agric. Sci., *34*, (1944):1.

[7]Folley, S. J., and Malpress, F. H.: The Response of the Bovine Ovary to Pregnant Mares' Serum and Horse Pituitary Extract. Proc. Roy. Soc., London, s.B., *132*, (1944):164.

[8]Casida, L. E., Warwirk, E. J., and Meyer, R. K.: Survival of Multiple Pregnancies Induced in the Ewe Following Treatment with Pituitary Gonadotrophins. J. Anim. Sci., *3*, (1944):22-28.

[9]Hammond, J., Jr.: Personal communication.

[10]Allen, E., Pratt, J. P., Newell, Q. V., and Bland, L. J.: Human Tubal Ova: Related Early Corpora Lutea and the Uterine Tubes. Contrib. Embryol., *22*, (1930):45.

[11]Hamilton, W. S., and Laing, S. A.: Development of the Egg of the Cow Up to the Stage of Blastocyst Formation. J. Anat., *80*, (1946):194.

Epizoötiology of Mastitis: The Relative Importance of Extended Exposure and of Age in the Spread of *Streptococcus Agalactiae* Infection

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THERE ARE numerous recorded observations of the higher incidence of streptococcic mastitis in older cows. Likewise, there is ample evidence that *Streptococcus agalactiae* has been the principal Streptococcus involved, and that it produces a chronic disease in which the infection usually persists from one lactation to the next. With each succeeding lactation, an additional number of cows, or quarters of cows, in a population become infected. These facts are compatible with the incremental pattern of incidence of this disease with advancing age, and suggested to early workers that extent, and perhaps degree, of exposure was a major factor in the build up of higher levels of incidence of this infection in older cows. On the basis of such an interpretation of the facts, mastitis control programs have usually contained provisions for the segregation and/or milking of infected animals last and the observance of certain sanitary practices in an attempt to reduce exposure of noninfected cows to *Str. agalactiae*.

Recently, Murphy [2, 3, 4, 5, 6] has proposed a new interpretation of this pattern of increase of incidence on age, namely, that more old cows are infected simply because more old cows have developed susceptibility. This susceptibility is supposed to be the expression of a specific change within the mammary gland itself, which change he has termed the "age factor." The age factor was postulated on the basis of data collected over a period of seven years on two herds of 60 cows each under the same management. Two observations relied upon in the development of the newer explanation were:

1) A close correlation was found between level of infection with streptococci and age[3]; and its corollary, that the level of infection in a herd was also correlated with the average age of the herd.*

2) Data were presented [1] which would imply that the incidence of staphylococcic mammary infections remained constant and did not increase on age.** It was reasoned from these observations that, since both staphylococci and streptococci share the teat canal as a common portal of entry, involvement of portal of entry alone could not be responsible for the increase of *Str. agalactiae* infections on age. The higher incidence of streptococcic infections in older cows was ascribed, therefore, to this acquired susceptibility, the age factor.

What Is the Age Factor?—For the purpose of the present discussion, several aspects of the age factor, as it is described, should be made clear: The age factor pertains to a supposed change within the mammary gland itself, rendering it susceptible to streptococcic infection.[2] The age factor becomes active in a regularly increasing number of a cattle population with the passing of time.[2] It develops independently of conditions of exposure.[2] Once acquired, the specific cows are thereafter susceptible to infection when, and if, exposed.[2]

Murphy has postulated that this age factor is the major predisposing factor to streptococcic mammary infection.[4] Anatomic considerations involving the teat meatus, degree of exposure, and prior sensitization were treated as being of minor importance.[4]

Testing the Validity of the Age Factor.—To merit acceptance, the hypothesis needs to be submitted to actual tests. There appear to be several tests or approaches that might be applied, some of which will be outlined here.

I—The Uniform Exposure Test.—One might establish a large group of cattle of all ages free of *Str. agalactiae* infection and subject them to a length of exposure which would be uniform in all age groups. Then, if older cows are more susceptible, the level of incidence in various age groups under uniform exposure should not differ materially from what is found in commer-

*Since this correlation might just as logically be applied to length of exposure and incidence, this observation is inadequate to support the new theory.

From the Department of Veterinary Science, University of California, Berkeley.

Presented before the Section on Research, Eighty-fifth Annual Meeting, American Veterinary Medical Association, San Francisco, Calif., August 16-19, 1948.

**Apparently, no effort was made to determine, according to present criteria, if the staphylococci were pathogenic. Moreover, a sample containing streptococci was classed only as Streptococcus infected, even though it might also have contained staphylococci. In our studies on staphylococcic mastitis, we have found a definite relationship between the incidence of infection with pathogenic staphylococci and length of time in the milking herd.

cial herds in which the disease has been enzoötic for several years.

However, if age per se is of little or no consequence, the level of incidence in various age groups under uniform exposure should be nearly equal.† In this instance the theory of an internal change (age factor) would be placed in doubt.

II—The Test of Permanency of Susceptibility.—For this test, it would be necessary to have a group of cows which had previously been infected with *Str. agalactiae*

STREPTOCOCCUS AGALACTIAE MAMMARY INFECTIONS

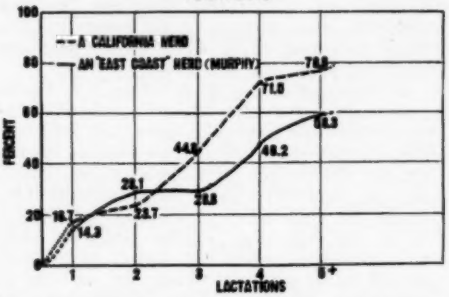

Fig. 1—A comparison of the incidence of streptococcic infections under extended exposure in the herd which provided data for the age factor hypothesis,† with the California herd which provided data used to test the validity of the age factor hypothesis. Of the two herds, the California herd showed greater bias toward higher incidence of infection in the oldest age group (76.6%), according to this initial survey made just prior to the introduction of a control program. Under normal herd conditions (extended exposure), cows in the fifth-and-over lactation group have received several times longer exposure than animals in the first lactation group (nine times as long in the strictly fifth lactation cows).

and cured by therapy, and a control group consisting of cows that had not previously been infected. Cows in the former group should be 100 per cent in possession of the age factor (having demonstrated susceptibility in a previous period of exposure) whereas, an undetermined number (depending on how long they had been kept independent of exposure) of those not previously infected should possess the factor.

If these two groups were then mingled and given equal opportunity for exposure, the group possessing the age factor should become infected nearly 100 per cent, while the group comprising a limited number of cows having the factor should have a distinctly lower incidence of infection.

III—The Quarter Distribution Test.—In this test, one could analyze the spread of infection by quarters in an exposed group of clean cows so handled as to provide uniform opportunity for infection.‡ All quarters of any one udder are subject to the same internal (physiologic, genetic, etc.) influences. If a change *within* the mammary gland occurs, it should occur in multiple quarters of the same individual; wherefore infection should tend to establish itself at the same time in multiple quarters of that individual. If, however, infection is brought about because of randomly distributed extrinsic influences, then single quarter infections should predominate over two- three- or four-quarter infections, at usual levels of incidence.

Our attention in this paper will be focused mainly on the first two approaches, the *uniform exposure test* and the *test of permanency of susceptibility.*

Source of Data.—The authors had an opportunity to test the age factor hypothesis in data collected on a commercial herd participating in a program for the control

DISTRIBUTION OF STREPTOCOCCUS AGALACTIAE INFECTION ON AGE (exposure being equal)

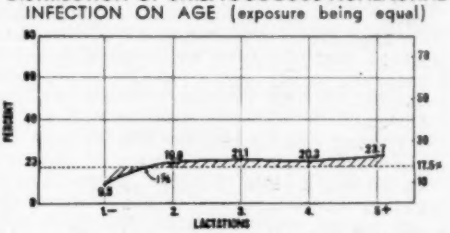

Fig. 2—Percentage incidence of infection observed after 629 clean cows of all ages were exposed to Streptococcus agalactiae, as described in the text. The increase beyond second lactation reaches a maximum of 4.7 per cent (from 19.0 to 23.7) in the oldest age group. The average percentage incidence in the population so exposed was exceeded at about 1 5/6 lactations on the graph.

of mastitis. On two separate occasions, conditions arose in this herd which resulted in the spread of *Str. agalactiae* among a large group of clean cows, some of which had been infected previously but cured by intramammary therapy.

The initial survey of this herd had revealed that of 182 milking cows exactly 50 per cent were infected with *Str. agalactiae.* The rate of incidence was 16.7 per cent of animals in the first lactation, 23.7 per cent of the second, 44.8 per cent of the

†Some bias on age should be expected because of the greater frequence of sphincter defects which have accumulated in older cows. Pendulous udders and chance injury and, perhaps, relaxed teat sphincter musculature are probably responsible for the loss of competence of the teat sphincter.

‡Milking order should be according to a predetermined pattern for "randomicity," or at least should be variable and with no regard for state of infection or age. Common managerial practices ordinarily effect inequalities in degree of exposure.

third, 71.5 per cent of the fourth, and 76.6 per cent of all animals fifth lactation and over. Staphylococcic mammary infections were also common but will not be discussed at this time.[§]

In the ensuing months, the program for eradication of *Str. agalactiae* was followed. The animals were treated, for the most part, with colloidal silver oxide while dry, penicillin being yet unavailable. In the meantime, the herd was being expanded by the addition of both raised and purchased replacements.

Outbreak Number 1.—At a time when the milking herd consisted of about 240 clean animals plus 60 suspicious and *Str. agalactiae*-infected animals (corralled separately and milked last), milking methods were changed from hand to machine. It was intended that machines be broken down and completely sterilized between milkings in order to preclude a carry over of organisms from the infected string which was milked last in each milking period to the clean cows milked first in the next milking period. After about five months, a spread of *Str. agalactiae* occurred in the clean strings. A check of management revealed that the source of spread could probably have been the inadequate sterilization of rubber parts, a duty recently transferred to a new employee. The outbreak occurred in winter months when corrals were muddy and chapped teats were common. It was estimated that the herd experienced a heavy exposure to *Str. agalactiae* for about four months, before adequate control was restored.

During the succeeding few months, by rigid adherence to a program of frequent bacteriologic tests of udder samples, segregation, intramammary therapy during the dry period, and slaughter, *Str. agalactiae* was completely eliminated from all animals of milking age. The herd then remained free of this pathogen for seventeen months.

Outbreak Number 2.—At the end of the seventeen-month period, *Str. agalactiae* was reintroduced into the milking herd. The source of infection was apparently a heifer which, seven days after being introduced in the herd, was proved infected with *Str. agalactiae*. On the day of the test, this heifer was the only animal found infected in the herd. She was removed three milkings later and brought to the University of California for observation. Testing of the herd at least once a week was recommended. The first case of spread was detected in a test eight days later; and, twenty days later, 6 more animals were found to be shedding *Str. agalactiae*. The owner at first attempted to restore the herd to a *Str. agalactiae*-free status by immediate slaughter of infected cows as they were detected. Twenty animals were removed during the first few weeks, but this procedure proved an economic burden. Thereafter, new cases were segregated as found and treated successfully with penicillin, which was then available for the first time.

Outbreak 2 also occurred in winter months and also lasted four months, but exposure was judged to be much lighter, since infected cows were more promptly removed from the main herd.

It was presumed that, since the heifer responsible for the second outbreak had been fed infective milk as a calf and had had ample opportunity to be suckled by pen mates, the infection had been introduced into its immature udder and persisted there until the time of first calving. Certainly, it is unlikely that, if the premises were the source of infection, such a large herd would have remained clean for as long as seventeen months. This emphasizes the need for proper calf management to protect heifers from infection and of testing all herd additions, regardless of source, before adding them to a clean herd.

Data.—To ascertain if this herd were peculiar in the respects (age and incidence of infection on age) being studied, we compared it with other herds as to age distribution (dispersion on age) and incidence of infection on age as determined in the initial survey. Table 1 gives the age distribution of cows in this analysis and shows that dispersion on age was quite comparable to other California herds. With respect to incidence of infection with *Str. agalactiae* in various age groups, the status of the herd prior to the control program is compared in figure 1 with the herd studied by Murphy and source of data on which the age factor hypothesis was based. Both show highest incidence of infection in the

TABLE 1—Comparison of Dispersion on Age. Per Cent of Total Herd in Various Lactation Groups

Lactation group	Per cent of total herd in each group	
	629 cows this analysis	Summary of cross sectional surveys in four California herds
1st	31.1%	28.2%
2nd	22.6%	23.5%
3rd	15.1%	16.1%
4th	13.4%	12.9%
over 4th	18.8%	19.3%

§Throughout these studies, all samples containing streptococci were studied by pure culture methods and the strains identified biochemically. (Details of methods used have been published.[5]) In this analysis, except where treatment or sale intervened, the minimum criterion for infection has been the finding of *Str. agalactiae* in two of three successive tests.

oldest cows, but the preliminary survey of the herd under study shows greatest bias toward high incidence in old cows.

Definitely increasing incidence on age existed prior to the control program in the herd providing our data, and there is no great departure from normal dispersion on age. Therefore, we should expect this herd to resemble other large dairy herds with respect to age and age susceptibility, and to respond typically to exposure to *Str. agalactiae*.

EXTENDED EXPOSURE IN RELATION TO SPREAD OF STREPTOCOCCUS AGALACTIAE

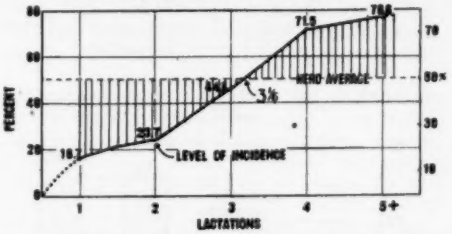

Fig. 3—Under normal conditions (extended exposure) prior to the initiation of a control program, average incidence of Streptococcus agalactiae infections in the herd being analyzed was exceeded at near the midpoint on exposure time or age. Compare with figure 2, where average incidence is exceeded far to the left of the midpoint on age.

The incidence of infection among 285 cows exposed in the first outbreak was 23.15 per cent, whereas, among 344 cows exposed in the second outbreak, it was 12.8 per cent. The over-all average incidence was 17.5 per cent (fig. 2).

Of the combined total (629 cows) exposed to *Str. agalactiae*, the precentage incidence of infection among cows in the various lactations was 9.6, 19.0, 21.1, 20.3, and 23.7 for the first, second, third, fourth, and over-fourth lactation groups, respectively. Taking the second lactation for a base because of certain spurious influences on the calculated incidence in heifers, there is a 4.7 per cent increased incidence with age over the range studied. Under extended exposure conditions, the incidence of infection in the oldest age group had reached 76.6 per cent.

In the outbreaks, there were 205 animals of second lactation and over which had been infected in earlier life and subsequently cured (hereinafter to be referred to as group P). There were 236 animals which had never been infected prior to the outbreaks (hereinafter to be referred to as group N). The average age of group P was 5.01 lactations; the incidence of reinfection was 21.95 per cent. The average age of group N was 3.53 lactations; the incidence of infection was 19.92 per cent. Thus, incidence of infection was only 2.03 per cent greater in the group of older, previously infected and cured cows (a group which, according to the age factor hypothesis, should have the age factor 100 per cent) than in the group composed of more younger cows which had never been infected.

I—The Age Factor Hypothesis Subjected to the Equal Exposure Test.—If the age factor were operative in a sufficiently greater proportion of older cows as to be responsible for the trend of increased incidence on age observed in figures 1 and 3, and if noninfected cows of all ages were given a uniform opportunity to become infected, a similar trend of increase in incidence on age should be reproduced.

In the present analysis, 629 clean cows were exposed to *Str. agalactiae* as described. Since all cows in the herd (with the exception of heifer additions during the outbreaks) had equal opportunity for exposure, exposure differs distinctly from that occurring in commercial herds where length of exposure is directly proportional to length of time in the milking herd. Therefore, the present data, figure 2, is adaptable to the equal exposure test with but few reservations. One such reservation is that referred to regarding heifers, part of which entered the herd for the first time at various intervals during the period of spread of *Str. agalactiae*.

COMPARISON OF INCIDENCE OF NEW STREPTOCOCCUS AGALACTIAE INFECTIONS

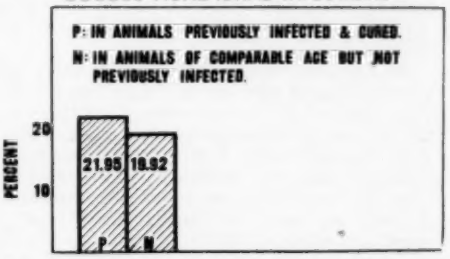

Fig. 4—Results of uniform exposure, in incidence of Streptococcus agalactiae infections in animals previously infected and cured, (P), and in animals never before infected, (N). Animals in group P, having earlier in life proved susceptible, should have possessed the hypothetical age factor 100 per cent. The 205 animals comprising group P had an average age of 5.01 lactations, whereas, the 236 animals in group N had an average age of 3.53 lactations. Previously infected animals expressed only 2.03 per cent higher susceptibility than younger animals never before infected.

Since first lactation animals were sub-

jected to spurious deviations in exposure and for other reasons, to be discussed separately, appeared to behave differently from the remaining groups, it was decided to confine further study to second lactation and older groups.

The data were surveyed to determine just what effect additional age beyond second lactation might have on susceptibility. Taking as a base the 19.0 per cent incidence of infection with *Str. agalactiae* which occurred in second lactation animals, it was seen that equal exposure of clean cows of all ages resulted in a maximum increase in incidence of 4.7 per cent in the oldest group (fig. 2). This slight increase under equal exposure is shown graphically in figure 5 in contrast with the normal increase on age observed in this herd prior to a control program. If an internal change with age were responsible for the trend normally observed on age, one might expect that the maximum increase under equal exposure should have been over 50 per cent (e.g., 76.6-19.0%).

Since results failed to simulate normal increase on age found where exposure is extended (fig. 1 and 3) and results resembled what might be expected if all age groups were about equally susceptible, we decided to submit the data to a statistical test of the latter assumption. Statistically, it appeared that age had practically no influence on susceptibility.* Extended exposure, rather than age, appears to be correlated with the increase which Murphy would attribute to an age factor.

Variations of this equal exposure test could be conducted by dividing the population on the basis of criteria other than age which, nevertheless, produce an age difference to ascertain if a correlation existed between age and infection. For example, the division of the herd into previously infected and cured (group P) and not previously infected animals (group N) results in groups with an age difference of 1.48 lactations (5.01 minus 3.53 lactations). According to the age factor hypothesis, increased age is associated with increased susceptibility. Prediction equations published suggest a differential in incidence of 26 to 31 per cent between the two groups.** The observed difference in incidence of infection between the two groups was only 2.03 per cent (21.95% minus 19.92%). This small difference might well be attributed to the expected greater number of patent or damaged teat meatuses normally encountered in older cows. The expected difference did not materialize, and there appears practically no correlation between age and susceptibility to infection.

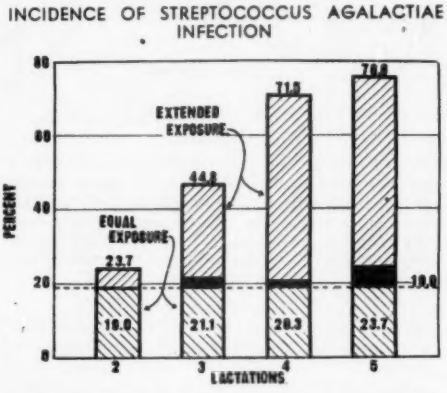

Fig. 5—Contrasting results of uniform exposure (black lines transecting vertical columns) to Streptococcus agalactiae, and of exposure which is extended in relation to milking age (full height of columns). Using the level of infection observed in second lactation animals under uniform exposure as a base (19.0 per cent, dotted line), the slight increase (maximum of 4.7 per cent in oldest age group) over the age range studied is depicted by the shaded areas. The light areas above show the added bias on age effected under normal herd conditions which provide extended exposure.

II—The Age Factor Hypothesis Subjected to the Test of Permanency of Susceptibility.—If infection is the consequence of an acquired internal change (age factor) then those animals which have become infected with *Str. agalactiae* should be regarded as those which possess the age factor. To select a group possessing the factor would simply call for the selection of animals with a history of having been infected. If such animals were then cured by therapy, one should then have a group 100 per cent in possession of the age factor to challenge by reëxposure. Group P is such a group, for the cows composing it were previously identified as susceptible to *Str. agalactiae* and later cured by intramammary therapy. Group N, composed of animals never before infected, should contain relatively few ani-

*The data were submitted to the following hypothesis: May the several age groups be considered, in so far as susceptibility is concerned, as random samples drawn from a population with a uniform probability of infection, in this instance, of 20.9 per cent?[9] The sum of chi square values was 0.90. The value of 0.90 will be exceeded in about 79 per cent of random samples drawn from a homogeneous population grouped for three degrees freedom. Thus, the hypothesis is far from being doubtful; there are no statistically significant deviations detectable among the four age groups tested for susceptibility.

**$1.48 \times 21.3826 \pm 5.7863$, or 31%, if Guernseys; or $1.48 \times 17.1874 \pm 4.1759$, or 26%, if Holstein-Friesians.[8]

mals possessing the age factor, since they had not long since been handled in an environment of exposure. With equal opportunity for exposure (fig. 4), group P became reinfected to a level of 21.95 per cent as compared with 19.92 per cent infection in group N.† Thus, those in which the age factor should have been uncommon were practically as susceptible as those theoretically 100 per cent in possession of the age factor. This suggests that susceptibility is related for the most part to temporary factors which are extrinsic and randomly distributed, rather than any internal change acquired with advancing age. In addition to the above tests of the age factor hypothesis, there are other observations which support the importance of exposure and tend to nullify the idea of importance of age.

1) Infection was greater in the first outbreak (23.15%) when exposure was judged to be heavy, than in the second outbreak (12.8%) when exposure stemmed from only 1 animal and infected animals were removed more promptly.

2) The annual increment in percentage incidence of infection shows no particular upward trend with age. A given fraction of clean animals, regardless of age, can apparently be expected to become infected during a year of exposure. This will be treated as a topic in a separate paper.

3) Data of others suggest that exposure is important. Plastridge,[7] for example, shows that heifers introduced into herds having a high incidence of infection with *Str. agalactiae* also have a high rate of infection in the first three months of the first lactation. This fact might be the outgrowth of degree of exposure, or of managerial or genetic factors common to the herd. None of these facts lend support to the idea that age per se is of much consequence, or that older cows are appreciably more susceptible.

SUMMARY

An analysis of records on University of California coöperators' herds was prompted by advent of the age factor hypothesis which states that with advancing age a change occurs within the mammary gland rendering it thereafter susceptible to streptococcic infection. Methods for testing the age factor hypothesis are outlined, and two are considered in this paper in detail.

The data include two outbreaks in a large herd, one of which occurred seventeen months after the herd had been freed of *Streptococcus agalactiae*. In the combined data, 629 animals were exposed to *Str. agalactiae* for a period of four months. Animals of all ages were involved, up to 14 years and 7 months, in the eleventh lactation.

Excluding first lactation animals because of spurious deviations, no significant relationship is found between susceptibility and age. Percentage incidence of infection was 19.0, 21.1, 20.3, and 23.7 for the second, third, fourth, and over-fourth lactation groups, respectively. The slight increase is attributed to the increased number of permanent anatomic changes involving the teat meatus to be expected in older cows.

Among the 629 animals exposed, there were 205 in second lactation and higher, which had been previously infected and cured some time prior to the outbreaks. Controls, which had never before been infected, included 236 animals over the same lactation range. The average age of the former group was 5.01 lactations, as compared with 3.53 lactations average in the group never before infected. According to prediction equations published with the age factor hypothesis, an age difference of 1.48 lactations should connote a difference in susceptibility, between these two groups, of 26 to 31 per cent. The actual difference in infection was found to be only 2.03 per cent, which is small enough to be accounted for by the increased number of animals with sphincter incompetence to be expected in the group containing a higher proportion of older cows.

The group previously infected and cured is, for having demonstrated its susceptibility, classed as having the age factor 100 per cent. However, when equal opportunity for exposure prevailed, only 21.95 per cent became infected. Yet, those in which the factor should have been infrequent had an incidence of infection of 19.92 per cent, only 2.03 per cent less. On the basis of these results, it is concluded that neither age per se, an age factor, nor a permanent change within the mammary gland play any important part in the spread of *Str. agalactiae*. Rather, extended exposure, to meet randomly distributed and transitory predisposing circumstances, is regarded as the major factor in the spread of this pathogen. Extended exposure, or length of time in the milking herd under exposed conditions, appears to be necessary to effect the bias toward higher incidence in older cows which the age factor hypothesis would attribute to age.

The inclusion of sanitary milking practices and segregation in programs for the control of *Str. agalactiae* is, therefore, regarded as sound, as it prevents the meeting

†The 2.03 per cent difference is explained above as possibly due to a greater number of permanent anatomic changes in the teat sphincters to be expected in group P because of its older average age.

of exposure to *Str. agalactiae* with the transitory, randomly distributed, predisposing circumstances permitting infection.

CONCLUSIONS

1) Clean old animals are practically no more susceptible to *Streptococcus agalactiae* infection than clean animals of second, third, and fourth lactations. It is suggested that, if animals with obviously incompetent teat sphincter mechanisms were excluded, strict equality would exist in all age groups.

2) Cows previously infected but cured of *Str. agalactiae* infection by therapy are but slightly, if at all, more susceptible to infection than those clean cows which have never before been infected.

3) Excluding cows with incompetent sphincter mechanisms, susceptibility is randomly distributed regardless of, and independent of, age and previous history of infection.

4) Conditioned by managerial and environmental conditions, a certain fraction of clean animals entering any lactation in an environment providing exposure to *Str. agalactiae* can be expected to become infected without regard for age.

5) Reduction of degree and extent of exposure are logical measures for the control of the spread of *Str. agalactiae*. To these should be added such managerial practices as will avoid trauma and injury to the mammary gland and particularly its portal of entry—the teat meatus.

References

[1]Murphy, James M.: The Genesis of Bovine Udder Infection and Mastitis. I. The Incidence of Streptococcal and Staphylococcal Infection in Relation to Predisposing Factors. United States Live-Stock Sanitary A., Proc. 49th Ann. Meeting, (1945) :30.

[2]Murphy, James M.: The Genesis of Bovine Udder Infection and Mastitis. III. Discussion of the *Age Factor* in Streptococcal (*Str. Agalactiae*) Infection. United States Livestock Sanitary A., Proc. 50th Ann. Meeting, (1946) :119.

[3]Murphy, James M.: The Genesis of Bovine Udder Infection and Mastitis. II. The Occurrence of Streptococcal Infection in a Cow Population During a Seven-Year Period and Its Relation to Age. Am. J. Vet. Res., *8*, (1947) :29.

[4]Report of 83rd Ann. Meeting A. V. M. A., Vet. Med., *41*, (1946) :379.

[5]Report of 51st Ann. Meeting United States Livestock Sanitary A. (1947), Vet. Med., *43*, (1948) :48.

[6]Murphy, James M.: The Posttreatment Occurrence of Streptococcal Infection in the Bovine Udder. Cornell Vet., *38*, (1948) :156.

[7]Plastridge, W. N.: Storrs Agric. Exper. Sta., Bull. 240, 1942.

[8]Schalm, O. W.: Merits and Deficiencies of Mastitis Diagnostic Methods. J. A. V. M. A., *105*, (1944) :398.

[9]Snedecor, George W.: Statistical Methods. Iowa State College Press, Ames, Iowa, 1940.

DISCUSSION

DR. J. O. SCHNAUTZ (Ore.): What was your method of exposure?

DR. ORMSBEE: Normal exposure. In the first outbreak, contaminated milking machines exposed the clean strings at the next milking. The second outbreak resulted from the introduction of a heifer which, on a herd test two days after she entered the milking herd, was found to be infected with *Streptococcus agalactiae*. She had been fresh seven days at the time. Of 344 animals which she exposed, 44 or 45 became infected. A total of 110 animals became infected in the two outbreaks, all as a result of normal exposure.

DR. K. R. PREUSSER (Calif.): Of the animals that became infected, what percentage had staphylococci present?

DR. ORMSBEE: We determined the presence of hemolytic staphylococci.

DR. PREUSSER: Which organism had the higher rate of infection?

DR. ORMSBEE: I can not answer that.

DR. W. G. PARKER (Pa.): What was your method of diagnosis?

DR. ORMSBEE: It has been described. It consisted of collecting introductory streams from every animal, plating the incubated samples, and picking colonies of streptococci for typing and identification. It was a pure culture study of every sample containing a Streptococcus.

VOICE: Was the heifer added to the herd accidentally?

DR. ORMSBEE: Yes.

VOICE: How long was it in the herd, and what precautions were taken?

DR. ORMSBEE: Seven days, under ordinary herd care.

DR. PREUSSER: What was the milking technique?

DR. ORMSBEE: Machines, without stripping, in a so-called milking parlor. Rapid milking procedure. The first outbreak occurred when part of the herd was milked as above, the remainder with ordinary bucket machines, but without stripping.

DR. PREUSSER: Were teat cups sterilized?

DR. ORMSBEE: No. Neither had the teats been dipped except during the first outbreak, when, for a few days, the teats were dipped in a cup of chlorine solution after milking.

DR. SCHALM: Please give us the figures for the days the heifer was in the herd.

DR. ORMSBEE: The heifer remained in the herd three milkings after being detected. Eight days later, 1 case was diagnosed; twenty days later, 6 new cases were found, and I do not recall further details.

CHAIRMAN MURPHY: What was the sanitation during that time?

DR. ORMSBEE: The sanitary practices, once the herd was cleaned up, actually did not amount to much. But, during the first outbreak, we made a strict effort to milk clean animals first and infected animals last. We autoclaved barn stools between milkings. For a time, milkers were asked to wash their hands in chlorine solution.

DR. PREUSSER: What was the incidence at various ages? Was a mastitis-control program in effect when the data were being collected?

DR. ORMSBEE: A minor effort had been made, for about a year prior to the program, to put heifers into the first strings milked and old cows, particularly the obviously infected ones, into the last strings. The plan was not strictly enforced, however.

This practice appears to have reduced infection about 7 per cent.

DR. PREUSSER: Was any treatment being used in the herd at the time?

DR. ORMSBEE: No. Later, silver oxide was used, mostly during the dry period. This was used to free the herd of *Str. agalactiae* infection the first time.

VOICE: Country practitioners do not have laboratory facilities available. What should they do to determine the type of infection present in the herd? What is a good program of prevention and treatment?

DR. ORMSBEE: Determining the type of infection may be a little beside the point of the present discussion.

VOICE: As a country practitioner, I determine the type of infection present by the character of the swelling found in the quarter(s), and the type of milk being secreted.

DR. HAVMAND (Copenhagen, Denmark): This case is very interesting. In Denmark, we find that infection tends to attain a balance within two years after the animal enters the milking

herd. This provides a significant age factor, because most of the animals enter as heifers. This factor of age at time of entering the herd may be in favor of the age factor theory.

DR. ORMSBEE: We find that of the 247 cases reported, the incidence does increase with age, but we believe this to be due to a longer and more severe exposure, rather than to an age susceptibility.

DR. HAVMAND: The time factor is significant, but in Denmark the program of hygiene is not high. Within a short time after entering the barn, a cow is exposed to infection, for streptococci are everywhere in the barn. We believe that the age factor is significant, and that time and injuries are not the complete explanation of the increased incidence.

CHAIRMAN MURPHY: From conversation with Dr. Havmand, I believe the question he raises is this: If testing had been continued in this herd, would the age relationship ever be established as found in normal herds untouched by artificial methods?

DR. ORMSBEE: Without a doubt, it would be. The average incidence would rise more rapidly at first and then level off because of the make-up of the herd.

CHAIRMAN MURPHY: If a straight line were to maintain itself, would it be mainly animals introduced or those already in the group for study which would become infected?

DR. ORMSBEE: We can not say what would happen, we can only presume, on the basis of the pattern of other diseases, that the highly susceptible animals will be the first to become infected. Hence, if there is an age susceptibility or predisposition, it should be apparent early in the outbreak.

DR. HAVMAND: Within four months?

DR. ORMSBEE: I think so. A high degree of susceptibility should express itself in that time.

DR. HAVMAND: We must consider both things. Time is a significant factor, and in the average herd may explain the entire problem. But we must also consider the number of lactation periods the animal has experienced before she became infected.

DR. ORMSBEE: That is shown in the graph. We cannot tell whether the animals all received full or equal exposure, nor whether the line would change in time. We postulate that the more susceptible animals were the first to be infected.

DR. HAVMAND: If 20 per cent of the heifers became infected, that is high, but not too high. And if 10 per cent of the treated animals were susceptible and became reinfected, the value of this figure depends upon the number slaughtered and the degree of selection exercised during or before treatment.

DR. ORMSBEE: Most of the slaughtering was done on a normal basis, but the damage to the udder was one factor in the decision.

VOICE: Is there a definite increase in the teat patency from the first to the fifth lactation period?

DR. ORMSBEE: I can not answer that. Dr. Murphy reports little importance from a family angle. However, experienced dairymen say that old cows milk more easily and in a larger stream than young cows. Whether this is due to teat patency or to a weeding out of the hard and slow milkers has not been determined.

DR. E. M. DOBBS (Calif.): Dr. Udall reports (*Cornell Veterinarian*, April, 1948) that injury to the teat sphincter is important. I believe that proliferations are increasingly important following the injury.

DR. PREUSSER: How did 20 per cent of the herd members become infected?

DR. ORMSBEE: At the time of the first outbreak, 15 per cent of the animals were infected. These were isolated and milked separately, so that animals becoming infected were presumably exposed by the milking machines.

In the second outbreak, there had been no infection in the herd for seventeen months preceding introduction of the infected heifer.

DR. PREUSSER: Were observations made on the number of injured teats among the infected and the clean animals in the herd?

DR. ORMSBEE: No.

DR. W. T. S. THORP (Md.): Would you care to discuss the effect of environment, milking methods, and general conditions (in so far as these might vary from one season to another) upon what you call susceptibility?

DR. ORMSBEE: Definite comparisons have not been drawn, but the spread was more rapid in winter than in summer. Part of this may have been due to chapped teats caused by mud that was often a foot or more deep.

DR. THORP: Was this taken into consideration when conclusions were drawn regarding susceptibility?

DR. ORMSBEE: The young and the old were kept in the same environment.

CHAIRMAN MURPHY: Can you tell us what happened from month to month as the disease spread and then was eradicated?

DR. ORMSBEE: At the outset, about 50 per cent of the cows were infected. This was brought down, through the months, to about 15 per cent. At this time, 285 cows were free from infection, and the infected animals (15%) were kept in isolation and milked last. During the first outbreak, infections increased to about 38 per cent but by concerted effort were brought back to zero, and the herd was free from infection for seventeen months.

In the second outbreak, each infected animal was removed immediately upon diagnosis, so that the level of infection remained low.

CHAIRMAN MURPHY: But at no time was the infection permitted to progress without interference and establish a normal level or balance. Therefore, your study and the one upon which I based my theory of increasing-with-age susceptibility are entirely different. The true factor of the age theory must be determined under natural conditions, when there is no treatment.

DR. ORMSBEE: We believe that our figures justify the conclusion that length of exposure, rather than increasing susceptibility with advancing age, account for the high level of infection in old cows.

CHAIRMAN MURPHY: We have no method of measuring rapidity of spread unless we permit the infection to proceed without interference to the stage of natural level. If artificial factors are introduced (treatment or removal of reactors), we do not know what to assume as a result.

DR. CECIL ELDER (Mo.): How can you be sure that during the so-called free period there were no carriers of a dormant infection?

DR. ORMSBEE: During the period of seventeen months, samples were drawn at about semimonthly intervals, and incubated in enriched medium. At no time did we get a culture of *Str. agalactiae* from any milk sample—until this heifer freshened.

DR. ELDER: After how many negative tests did you assume an animal to have recovered?

DR. ORMSBEE: Only after milk samples had been negative for an entire lactation period.

DR. PREUSSER: Was the heifer introduced from another herd?

DR. ORMSBEE: No. It was raised in the herd during the first outbreak. Eight heifers were kept in one pen, and fed milk from the infected animals. Since there was opportunity, we assume that this animal was infected as a calf, by having other calves suck her udder.

DR. F. E. MARTIN (Pa.): Do you have data on the presence of streptococci or staphylococci in acute, subacute, and chronic cases?

DR. ORMSBEE: No precise data. The incidence of acute infection was higher in the fresh cows, and this was associated with *Str. agalactiae*. The incidence was never great during the program.

The Response of Cattle to Penicillin Preparations Following Intramuscular Injection

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THE EFFICACY of penicillin therapy depends upon the maintenance of concentrations of penicillin in the tissues, lymph, and blood. Subbacterial or subbacteriostatic concentrations of the antibiotic may produce, in some cases, resistant strains of the infecting microörganism. The presence of traces of penicillin in the blood may well represent much higher concentrations in the lymph and tissues. Schachter[1] found the lymph penicillin level in dogs to be a more reliable index of therapeutic efficacy than the blood level. He felt the lymph level tent of the lymph was greatly diluted. The drug was again evident in the urine after concentration and excretion by the kidneys. In the tables accompanying this article, trace levels of penicillin in the blood are recorded; they may well be very significant in the light of this recent evidence.

Ercoli *et al.*[2] found, in hemolytic streptococcal and Type I pneumococcal infections in rats, that sufficient penicillin remains in the host, after the blood levels have fallen to zero, to offer protection to the host in many cases for six to eight hours. Eagle

TABLE I—Blood and Urine Levels of Penicillin in Cattle*

	1,000,000 I. U.							
Hours after injection	Cow 1: 800 lb.		Cow 2: 1,000 lb.		Cow 3: 1,000 lb.		Cow 4: 1,000 lb.	
	Plasma level**	Urine level**	Plasma level**	Urine level**	Plasma level**	Urine level**	Plasma level**	Urine level**
4	0.0312	20.0	>0.0312	20.0				
6					0.0625	>40.0	>0.0625	80.0
8	>0.0312	20.0	>0.0312	20.0				
12	0.0312	20.0	0.0625	20.0				
20					0.0312	80.0	0.0312	>20.0
24	0.0312	40.0	0.0312	20.0				
30					0.0156	>10.0	>0.0156	20.0
36	>0.0312	20.0	0.0156	20.0				
44					0.0156	1.25	trace	> 2.5
48	0.0156	10.0	trace	10.0				
54					trace	1.25	trace	1.25
60	trace	5.0	trace	10.0				
68					0.0156	0.312	trace	1.25
72	trace	2.5	trace	5.0				
78					0.0156	0.156	0.0	0.625
84	0.0	1.25	0.0	5.0				
92					trace	0.156	trace	0.312
96	trace	>0.625	trace	2.5	trace	0.0156	trace	0.156
100	0.0	0.625	0.0	2.5				
102					0.0	0.0	0.0	0.0625
104	0.0	0.625	0.0	>1.25				
108	trace	0.625	trace	2.5				
116					0.0	trace	0.0	trace
120	0.0	0.312	0.0	1.25				

*Procaine penicillin suspended in peanut oil containing 2% aluminum monostearate intramuscular injection.
**Expressed as I. U./cc.

closely resembled tissue concentrations of the drug. Penicillin was frequently present in tissues when the blood level was zero. As the lymph left the thoracic duct and entered the venous circulation, the penicillin con-

et al.[3] reported that, in experimental syphilis in rabbits, sustained, small concentrations of penicillin in the blood were more effective than high levels for shorter periods. They felt the repository preparations accomplished the same results as multiple small doses.

Several papers have appeared which re-

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TABLE 2—Blood and Urine Levels of Penicillin in Cattle*

	1,500,000 I. U.				
Hours after injection	Cow 1: 1,000 lb.		Cow 2: 700 lb.	Cow 3: 1,000 lb.	
	Plasma level**	Urine level**	Plasma level**	Plasma level**	Urine level**
12	0.125	>20.0			
24			>0.0312	0.0625	40.0
36	trace	1.25			
48			0.0156	trace	1.25
60	trace	0.0625			
72	trace	0.312	trace	0.0	0.312
84	0.0	0.125			
96	0.0	0.125	trace	0.0	0.312
120			trace	0.0	0.0

*Procaine penicillin suspended in peanut oil containing 2% aluminum monostearate intramuscular injection. **Expressed at I. U./cc.

port penicillin blood levels attained in horses.[4,5,6] Relatively few data have been published for cattle.[7,8] It has been felt worthwhile to make these determinations of blood and urine penicillin levels in cattle, using several penicillin preparations which are now available to the practitioner to provide data which could be used as a dosage guide for this species.

MATERIALS AND METHODS

All of the cattle used in this study were apparently normal animals, unless otherwise noted in the tables. Only dairy breeds were used. The following penicillin preparations in various dosages were used: aqueous solutions of penicillin, the Romansky formula, procaine penicillin G in oil, and procaine penicillin suspended in peanut oil containing 2 per cent aluminum monostearate (w/v).** The last three products contained 300,000 I. U. per cubic centimeter. Only intramuscular injections were employed.

All assays of blood plasma and urine were made in accordance with the technique of R. J. Hickey,[9] using *Bacillus subtilis* as the test organism. Penassay broth (Difco) was the nutrient medium used in the serial dilutions. The final reaction of the medium approximated pH 7.0. Tests were made in triplicate or quadruplicate. All discrepancies in a series were rechecked in the same manner.

**This product was supplied by Bristol Laboratories, Syracuse 1, N. Y.

RESULTS

Blood and urine levels attained after the injection of the respective products, using various dosages, are given in the text or shown in the tables, or both.

CONCLUSIONS

1) Crystalline penicillin G in aqueous solution gave high blood and urine levels for short periods. A dosage of 300,000 I.U. was detectable for three hours in the blood and for seven hours in the urine. One million I.U. gave a blood level of greater than 0.0156 I.U. for a five-hour period. At the end of seven hours, penicillin was still present in traces in the blood and at a 10-I.U. concentration in the urine. The need of repeated administration greatly limits the use of such a product.

2) The Romansky formula (penicillin in oil and beeswax), when 1 million I.U. were injected, exhibited a blood level of at least 0.0156 I.U. for twenty hours and a minimum urine level of 10 I.U. for thirty-six

TABLE 3—Blood and Urine Levels of Penicillin in Cattle*

	2,000,000 I. U.				
Hours after injection	Cow 1: 1,000 lb. Two injection sites Plasma level**	Cow 2: 800 lb. Two injection sites Plasma level**	Cow 3: 850 lb. One injection site Plasma level**	Cow 4: 1,000 lb. One injection site Acute metritis case	
				Plasma level**	Urine level**
24	0.0625	0.0625	0.0312	0.0625	10.0
48	0.0156	>0.0156	>0.0156	0.0312	20.0
72	trace	trace	trace	0.0312	
96	trace		0.0	trace	2.5
120	0.0		0.0	trace	5.0

*Procaine penicillin suspended in peanut oil containing 2% aluminum monostearate intramuscular injection. **Expressed as I. U./cc.

hours. A dose of 1.5 million I.U. gave essentially the same levels for twenty-two hours, and traces of the drug remained for forty-eight hours in both blood and urine. The slow absorption of the beeswax in this product is a decided disadvantage in valuable animals. At the dose levels employed, this product is probably effective for about twenty-four hours.

3) Procaine penicillin G in oil gave blood levels of at least 0.0156 I.U. for twenty-six to twenty-eight hours, when a dose of 1 million I.U. was used. Traces of penicillin were observed in the blood at the end of fifty-six hours. A minimum urine level of 10 I.U. was noted for thirty-seven to fifty-six hours in the 2 cows. A 300,000-I.U. dose produced detectable amounts of the drug in the blood and a 10-I.U. level in the urine for ten hours. So far as maintenance of blood level is concerned, this product acts quite similarly to the Romansky formula.

One million I.U., repeated every forty-eight hours, did not produce the high penicillin blood levels that had been anticipated. Three million I.U., when administered twice with an interval of seventy-two hours between injections, resulted in a 0.0312-I.U. level for a period of ninety-six hours after the second injection. Trace amounts were observed at the end of 120 hours.

This product produced no obvious irritation when 10-cc. amounts were injected at one site in the large caudal thigh muscles. It was found that better blood and urine levels were maintained when the entire dose was deposited at one site than when it was divided and two sites of injection used.

It is quite possible that this product may be effective for ninety-six to 120 hours when one considers the *in vivo* studies of Ercoli and Eagle. High blood levels may be expected at the 3 million-dose level for seventy-two to ninety-six hours. This product

TABLE 4—Blood and Urine Penicillin Levels in Cattle*

	3,000,000 I. U.					
	Cow 1: 1,000 lb.		Cow 2: 1,000 lb.		Cow 3: 1,000 lb.	
	Two injection sites		Two injection sites		Two injection sites Pyelonephritis case	
Hours after injection	Plasma level**	Urine level**	Plasma level**	Urine level**	Plasma level**	Urine level**
24	>0.0625	80.0	0.125	40.0	>0.0312	40.0
48	trace	2.5	>0.0156	10.0	0.0312	20.0
72	trace	0.25	trace	2.5	0.0312	2.5
96	trace	0.125	0.0156	0.625	0.0156	> 5.0
120	0.0	0.0	0.0	0.0	0.0156	5.0

Hours after injection	Cow 4: 850 lb. Two injection sites Plasma level**	Cow 5: 1,000 lb. Two injection sites Plasma level**	Cow 5: 1,000 lb. Two injection sites Urine level**	Cow 6: 850 lb. Two injection sites Plasma level**	Cow 7: 850 lb. One injection site Acute metritis case Plasma level**	Cow 8: 850 lb. One injection site Acute metritis case Plasma level**
24	0.125	0.0312	40.0	>0.0312	0.0312	0.0625
48	0.0312	0.0312	40.0	>0.0625	>0.0312	0.0312
72	0.0156	0.0312	40.0	>0.0156	>0.0156	0.0312
96		0.0156	20.0	trace	trace	trace
120		0.0	20.0	0.0	trace	trace

*Procaine penicillin suspended in peanut oil containing 2% aluminum monostearate intramuscular injection.
**Expressed as I. U./cc.

4) Procaine penicillin suspended in peanut oil containing 2 per cent aluminum monostearate gave good blood and urine levels for a thirty-six-hour period, when a dose of 1 million I.U. was used. The administration of 1.5 million I.U. produced essentially the same results. Two million I.U. gave good blood and urine concentrations of penicillin for forty-eight to seventy-two hours. Detectable amounts of penicillin were present in both the blood and urine at the end of ninety-six hours; traces of penicillin were evident after 120 hours, in some cases.

renders unnecessary the repeated administration of penicillin, and, for this reason, it should find wide application.

References

[1]Schachter, R. J.: Fate and Distribution of Penicillin in the Body. I. Circulation of Penicillin in the Lymph. Proc. Soc. Exper. Biol. and Med., 68, (1948):29-34.

[2]Ercoli, N., Lewis, W. M., Schwartz, B. S., and Whitehead, M.: Fate and Distribution of Penicillin in the Body. II. Duration of Blood Concentrations and Chemotherapeutic Effectiveness. Proc. Soc. Exper. Biol. and Med., 69, (1948):297-299.

[3]Eagle, H., Magnuson, H. J., and Fleishman, R.

The Effect of the Method of Administration on Therapeutic Efficacy of Sodium Penicillin in Experimental Syphilis. Bull. Johns Hopkins Hosp., *79*, (1946):168-189.

[4]Doll, E. R., and Dimock, W. W.: Penicillin Dosage and Blood Levels for Horses. J.A.V.M.A., *108*, (1946):209-214.

[5]Doll, E. R., and Dimock, W. W.: Efficacy of Various Agents for Delaying Absorption of Penicillin. J.A.V.M.A., *108*, (1946): 310-313.

[6]Doll, E. R., and Wallace, M. E.: The Blood Level Response of Horses to the Administration of Penicillin in Oil and Wax. J.A.V.M.A., *113*, (1948): 240-244.

[7]Beck, J. B., De Mott, T., and Boucher, W. B.: A Report of Six Cows with Infectious Cystitis and Pyelonephritis Treated with Penicillin. Vet. Extension Quart., Univ. of Pennsylvania, *100*, (1945): 10-16.

[8]Morse, E. V.: Bovine Pyelonephritis. Cornell Vet., *38*, (1948):135-147.

[9]Hickey, R. J.: Serial Dilution Assay Method for Penicillin in Blood and Other Body Exudates Employing *Bacillus Subtilis*. Mimeographed Paper, (1945):1-5.

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Helen S. Bayless, Notary Public (commission expires October, 1953)."

The Effects of Large Doses of Various Sulfonamides Injected Intravenously in Dairy Cattle

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THE OBJECT of this experiment was to determine the tolerance of dairy cows to large single doses of various sodium sulfonamides injected intravenously. The experiment developed following a report by Forman *et al.*[1] on the use of large single intravenous injections of sulfapyridine sodium in the treatment of interdigital phlegmon (foot rot) in dairy cattle. Forman used 60-Gm. and 90-Gm. doses on all dairy cows, irrespective of weight. Hence, in this experiment, the usual dosage to body weight relation was disregarded. Most of the experimental cows weighed from 800 to 850 lb. and were fairly uniform.

PLAN OF THE EXPERIMENT

Six different sulfonamides were studied. Eighteen mature dairy cows of mixed breeds, divided into six groups of 3 each, were injected intravenously with the water-soluble sodium salt of a sulfonamide. The kind and amount of drug injected into each cow and the number of injections are shown in table 1. The drugs were weighed out as the free sulfonamides and then converted to a soluble sodium sulfonamide by adding the necessary and minimal amount of sodium hydroxide to a suspension of the free drug in about 500 cc. of water. This procedure was followed because of the lack of published information on the amount of water of crystallization present in a gram of the sodium salt of each sulfonamide. The intravenous injections were made with a gravity-flow apparatus and a 14-gauge hypodermic needle.

Table 1 shows that each group of 3 cows was treated as a separate unit and was used to study the effects of only one drug. All groups underwent similar treatment, with the exception of the one devoted to sulfaquinoxaline. The procedure was altered for the latter group because, after the experiment started, it became apparent that the dosage of sulfaquinoxaline employed was excessive.

After all 3 animals in each group were injected intravenously with 60 Gm. of sulfonamide, numerous blood samples were drawn at scheduled intervals. Analyses of these samples gave information on the blood-sulfonamide concentration, the blood cell and hemoglobin picture, and the blood urea-nitrogen concentration.

The blood samples were analyzed for sulfonamide content by the method of Bratton and Marshall.[2] It was suggested* that the method be checked to see if a part of the sulfaquinoxaline was precipitated along with the proteins of ox blood by the addition of trichloracetic acid. The data in table 2 resulted from three different trial analyses with known quantities of sulfaquinoxaline in ox blood.

From the data of table 2, it was apparent that sulfaquinoxaline was different from other sulfonamides when analyzed quantitatively by the standard method of Bratton and Marshall. The data showed that only about 76 per cent of the original amount of sulfaquinoxaline was

*The suggestion was made by D. F. Green from analysis of unpublished data dealing with the use of sulfaquinoxaline in chickens.

TABLE 1

THE KIND AND AMOUNT OF SULFONAMIDE INJECTED INTRAVENOUSLY INTO DAIRY COWS

DRUG *	COW NO.	GMS. INJECTED INTRAVENOUSLY 1	2	3	4
SULFATHIAZOLE	10	60			
	12	60	60		
	11	60	60	90	90
SULFAPYRIDINE	14	60			
	15	60	60		
	13	60	60	90	90†
SULFADIAZINE	93	60			
	65	60	60		
	74	60	60	90	90
SULFAMERAZINE	21	60			
	20	60	60		
	19	60	60	90	90
SULFAMETHAZINE	18	60			
	16	60	60		
	17	60	60	90	90
SULFAQUINOXALINE	58	60			
	35	60	48		
	89	60†			

* Converted to Sodium Salt
† Death

From the Division of Veterinary Medicine, Iowa State College, Ames (Jones and Smith); from the Division of Veterinary Medicine, University of Minnesota, St. Paul (Roepke).

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The aid and coöperation of the Meat Inspection Division of the Bureau of Animal Industry, Des Moines, Iowa, in inspecting the experimental cows at slaughter and in providing tissues from them were appreciated.

The courtesies and coöperation of the owners and management of the Bookey Packing Co. were appreciated in securing and salvaging the experimental animals.

present when the final reading was taken. Therefore, 76 per cent was used in this report as a correction factor in calculating all data on sulfaquinoxaline.

TABLE 2—The Measurement of Sulfaquinoxaline in Ox Blood by the Bratton and Marshall Method: An Average of Three Trials

Known amount of sulfaquinoxaline in mg. per 100 cc. of ox blood	Amount of sulfaquinoxaline present in filtrate	
	Mg. per 100 cc. of ox blood	Per cent of known amount
2.5	1.87	74.8
5.0	3.965	79.3
10.0	7.27	72.7
15.0	11.3	75.3
20.0	15.75	78.7
	Average	76.16

Clinical observations were recorded during and following the administration of each drug. Five days after administering the drug, 1 cow from each group was sent to slaughter. These animals were examined carefully at autopsy. The entire urinary tract and a section of the liver were returned to the laboratory for detailed study. The cows were slaughtered as indicated in table 1. Intervals of seven to ten days lapsed from the end of one trial to the beginning of another trial in the same animal.

The cows were confined to stanchions in a well-constructed cattle barn, where they were cared for in the customary manner. Twelve of the cows were under observation for two weeks before the experiment began. The other 6 cows were under observation for only three days because of difficulty in obtaining them according to plan.

CLINICAL OBSERVATIONS

First Trial.—Cow 17, receiving sulfamethazine sodium, showed drug "shock" after about 48 Gm. of drug had been injected. The reaction was characterized by temporary blindness accompanied by a "starey" look, muscle tremors, and apparent muscular weakness, because the head was held low and the legs were wide apart. The cow fell to the floor when the halter rope was released. Within six to eight minutes, she struggled to her feet and, ten minutes later, the remaining 12 Gm. of sulfamethazine sodium was injected with no difficulty.

Cows 58, 35, and 89 showed drug shock after each received 60 Gm. of sulfaquinoxaline sodium. The administration of 60 Gm. proved to be an excessive dose but, due to lack of information regarding the drug, this could not be anticipated. All 3 cows exhibited severe muscle weakness, interference in sight, and a thoroughly dazed and helpless attitude. Judged clinically, the reaction seemed to arise from an acute toxic effect upon the central nervous system. Respiration and heart action did not seem to be altered radically. Cow 58 showed an improved reaction to external stimuli one hour after the injection. Within another hour, she tried unsuccessfully to stand up. About four hours after the injection, cow 58 stood up in the stanchion and drank water. At the same time, the remaining 2 cows in the group, 35 and 89, were largely unchanged. The cornea of these cows could still be touched without provoking a reflex movement. Cow 35 swallowed water with difficulty and cow 89 did not drink. Eleven hours after injection, cow 35 got to her feet. From this time until her death on the ninth day after treatment, cow 89 struggled unsuccessfully to rise to her feet. She could move her legs into the proper position and attempt to get up, but her legs were never able to lift her body. She ate and drank moderately well, ruminated, and responded to most external stimuli. One day before her death, a bed sore opened on her brisket, revealing considerable necrosis of the tissue.

Second Trial.—The second injection of 60 Gm. was administered intravenously to the 2 cows remaining in each group, with 1 exception. In the group for study of sulfaquinoxaline, 1 cow had died, 1 had gone to slaughter, and the 1 cow remaining received only 48 Gm. of drug.

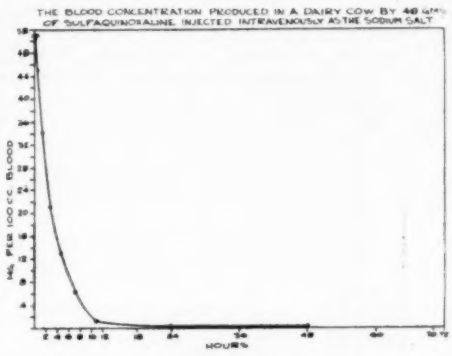
THE BLOOD CONCENTRATION PRODUCED IN A DAIRY COW BY 48 GMS. OF SULFAQUINOXALINE INJECTED INTRAVENOUSLY AS THE SODIUM SALT
MG. PER 100 C.C. BLOOD
HOURS

Figure 1

No significant observations were noted in any except cow 35, which received the 48 Gm. of sulfaquinoxaline. Again, drug shock was observed at the close of the intravenous injection. The cow leaned against her stanchion for support and fell down twenty minutes after administration of the drug. She managed to get onto her feet six to seven hours later and seemed to feel all right thereafter.

Third Trial.—The third series of intravenous injections were made into the 1 cow remaining in each of five drug groups with only one minor incident. After about 60 Gm. of sulfadiazine sodium were injected into cow 74, administration was halted because of muscular tremors and weakness, as well as an irregular and feeble respiratory pattern. This reaction soon passed and administration was completed.

Fourth Trial.—The fourth series of injections were completed without incident. However, cow 13 showed a nervous reaction two hours after the injection of 90 Gm. of sulfapyridine sodium. She was nervous and restless; drank a few swallows of water every three to five minutes; dipped her nose into the water repeatedly between swallows; and had a shallow and fast respiration. Five hours after injection, cow 13 showed marked depression, rapid and shallow respiration, and severe hematuria. Blood dripped from punctures of the jugular vein made two hours earlier. The cow continued to bleed from the jugular punctures until sometime during the night. A sample of blood collected in a test tube formed no clot in twenty-four hours. The cow became very weak and depressed and respiration was rapid and labored. The temperature was normal until the next morning, when it was only 99 F. By this time, cow 13 was very depressed and it seemed probable that she would die soon. A sample of blood from the mammary vein clotted in eight minutes. She died thirty hours after receiving the intravenous injection.

Observations on Body Temperature.—The rectal temperature of each cow was determined twice daily, beginning on the day preceding the injection of drug and continuing as long as blood samples were taken. No significant variations in temperature were noted. Three cows showed a 1-degree (F.) rise above the previous maximum about sixty hours after receiving a dose of sulfonamide, but this rise was attributed to some other cause.

Sulfaquinoxaline and the Blood-Clotting Time.—Other work has indicated that sulfaquinoxaline might dangerously prolong the blood-clotting time. Therefore, samples of freshly drawn blood were measured by the glass capillary-tube method for blood-clotting times. The results are shown in table 3.

From table 3, it is apparent that sulfaquinoxaline temporarily prolongs the blood-clotting time in dairy cows. This prolongation, however, produced no unfavorable reaction apparent from the clinical standpoint.

Blood Cells and Hemoglobin.—A total of

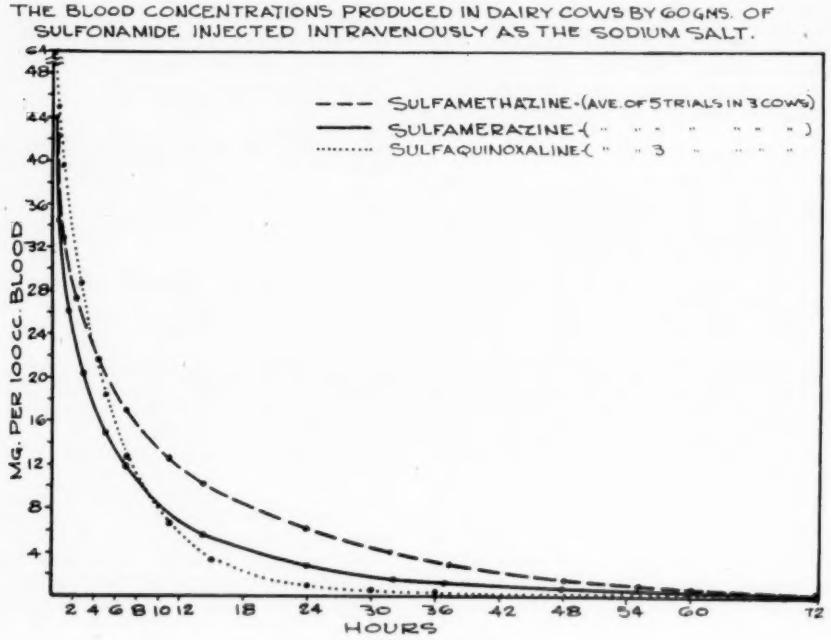

Figure 2

four or seven or 12 blood cell counts were made on cows in each group, depending upon the date of slaughter. No significant variations were observed in the total number of red cells with the exception of cow 13, which died with internal hemorrhages. Cow 13 showed a marked and progressive decrease in red blood cells immediately before death.

TABLE 3—Influence of Sulfaquinoxaline on the Blood-Clotting Time of Cows

Time	Cow 35	Cow 58	Cow 89
7-18-48	7 min.	7 min.	7 min.
7-19-48—8 a.m.	60 Gm. intravenously		
7-19-48—4 p.m.	14 min.	13 min.	10 min.
7-20-48—8 a.m.	16 min.	7 min.	11 min.
7-21-48—4 p.m.	8 min.	9 min.	18 min.
7-22-48—4 p.m.	8 min.	7 min.	12 min.
7-23-48—8 a.m.	.....	.....	6 min.
7-24-48—8 a.m.	7 min.	.....	.....

The total white blood cells in most cases varied little or none during the experiment. Where slight variation occurred, there was no consistent trend, except in those cows that received sulfapyridine and 1 that received sulfaquinoxaline. The intravenous injection of sulfapyridine sodium appeared to cause a brief, slight increase in total white cell and in the polymorphonuclear counts. Cow 89, which received 60 Gm. of sulfaquinoxaline, showed an increase in total white count that was about two and one-half times the normal. The increase occurred almost entirely in polymorphonuclear leucocytes. The white cell count of cow 89 remained abnormal until her death ten days following the administration of drug.

No significant variations were observed in successive measurements of grams of hemoglobin in any of the animals other than cow 13. At the time of the fourth and last injection of sulfapyridine sodium into cow 13, there were 10.1 Gm. of hemoglobin per 100 cc. of whole blood. This figure decreased to 6.55 Gm., twenty-four hours later. Death followed within about four hours.

Blood-Urea Nitrogen.—A total of 147 blood samples from the 18 cows were analyzed for urea-nitrogen content. The minimum reading was 5 mg. per 100 cc. of whole blood and the maximum was 25 mg. The range common to most of the samples was 6 to 12 mg. During her prolonged illness, cow 89 showed a progressive increase in blood-urea nitrogen to a maximum figure of 25 mg. Cow 13, which died of internal hemorrhages, did not show an increased blood-urea nitrogen. None of the remaining cows showed significant variations from normal in the urea-nitrogen content of the blood.

Blood-Sulfonamide Concentrations.—The varying sulfonamide-blood concentrations produced by the intravenous injections are clearly shown in figures 1 to 5.

Of considerable clinical importance is the duration of sulfonamide concentrations in the blood stream following a single intravenous injection. Table 4 was compiled from figures 1 to 5 to facilitate a more exact comparison of the concentrations of the six different sulfonamides within the blood stream. Two concentrations were arbitrarily chosen in order to compare the approximate duration of therapeutic activity from single doses of sulfonamides administered intravenously. In table 4, are listed the respective number of hours during which the blood-sulfonamide concentrations were above the minimal figures of 5 mg. per 100 cc. of blood and 2 mg. per 100 cc. of blood following the intravenous administration of sulfonamides. After the 60-Gm. doses, the drugs disappeared in the following order from the blood stream: sulfathiazole, sulfaquinoxaline, sulfadiazine, sulfamerazine, sulfapyridine, and sulfamethazine. After the 90-Gm. doses, the order of disappearance was the same, except that sulfapyridine lasted as long as did sulfamethazine.

TABLE 4

THE DURATION OF BLOOD SULFONAMIDE CONCENTRATIONS IN DAIRY COWS FOLLOWING INTRAVENOUS INJECTIONS.

DRUG	60 Gm. DOSE MAINTAINED BLOOD LEVEL IN EXCESS OF		90 Gm. DOSE MAINTAINED BLOOD LEVEL IN EXCESS OF	
	5 mg. / 100 c.c.	2 mg. / 100 c.c.	5 mg. / 100 c.c.	2 mg. / 100 c.c.
SULFATHIAZOLE	5 Hours	8 Hours	8 Hours	15 Hours
SULFAQUINOXALINE*	13	18	—	—
SULFADIAZINE	14	21	15	21
SULFAMERAZINE	17	29	18	32
SULFAPYRIDINE	23	38	33	46
SULFAMETHAZINE	29	44	33	46

* A dose of 48 Gms. maintained the blood level in excess of 5 mg./100 c.c. for 8 hours and of 2 mg./100 for 10 hours.

PATHOLOGY

The following data covered 18 animals, including 2 that died—for which necropsy reports will follow. Tissues from 1 animal were not available.

The experimental cows were slaughtered in a commercial packing plant at the times indicated under "Plan of the Experiment." The animals were examined antemortem and postmortem, in the routine manner, by inspectors of the U. S. Department of Agriculture. All of the cows passed inspection and were apparently normal in all respects, except as noted hereinafter. The complete

urinary tract and a piece of liver were returned to the laboratory for detailed examination.

After the first trial, a few small calculi were found in the urinary bladders of cows 18 and 21. They apparently were not sulfonamides, however, because they did not give a positive reaction when isolated and tested. A few dark brown calculi were found in the pelvis of one kidney in cow 58 and also cow 93, but none were sulfonamide in nature.

Following the second trial, cow 15 had a large amount of ingesta in her rumen, as if she had suffered a digestive disturbance. The right lung showed slight consolidation and the liver revealed a localized fibrosis. Both kidneys from cow 35 contained yellow precipitated crystals of sulfonamide in the collecting tubules of all the renal pyramids and in the tubules of the renal cortex, also. In cow 65, a small area on each apical lobe of the lungs showed consolidation. A suggestion, grossly, of precipitated sulfonamide crystals were observed in the renal-collecting tubules of one kidney from cow 65.

There were no necropsies after the third trial. Following the fourth trial, 3 cows were free from abnormalities. A fourth cow (74) passed inspection but, through error, the urinary tract and a part of the liver were not available. Cow 13 died after the fourth injection of sulfapyridine sodium.

Kidneys.—All kidneys showed acute passive congestion involving the venules of the medulla; about one third showed albuminous casts in the tubules, sometimes accompanied by mild calcification in the pyramids. In about one half, there was loss of the superficial portion of the cytoplasm in the proximal convoluted tubules, a change somewhat akin to that which results from certain metallic poisonings, but milder. The preceding can reasonably be attributed to the sulfonamides. In addition, some 60 per cent of the kidneys showed mild glomerular changes, such as increased cellularity, the significance and importance of which seem questionable.

Quite puzzling is the fact that the kidneys of several (about one third) of these cows showed localized changes of an apparently infectious nature in the form of cellular infiltrations and, in some cases, pus in certain scattered tubules. Such changes were mild and localized, but cannot be ignored. Other renal changes included petechial hemorrhages in 1 and hemosiderosis in another.

Livers.—All the livers showed cloudy swelling and albuminous degeneration,

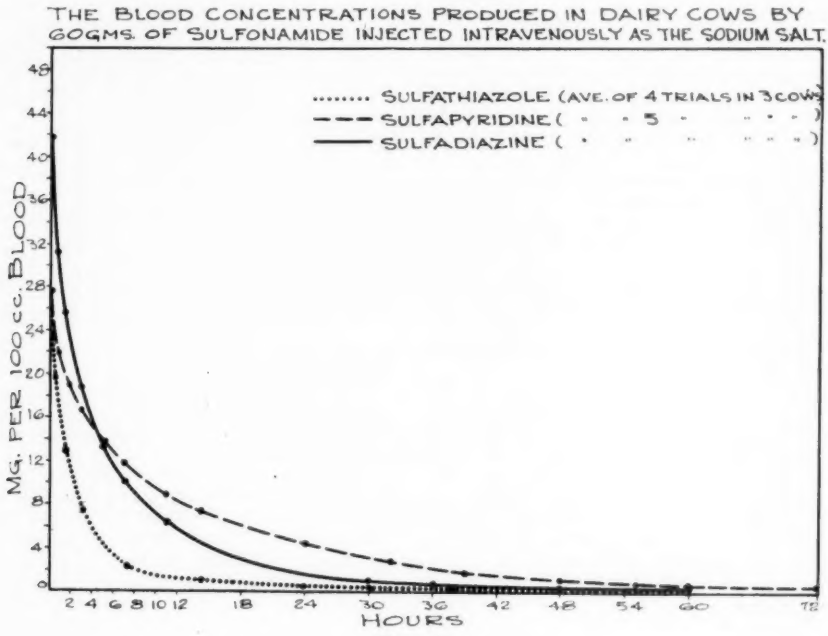

Figure 3

sometimes with an advancing necrosis of hepatic epithelium and sometimes accompanied by intracellular fat. These are all in the nature of a toxic degeneration which can doubtlessly be attributed directly to the sulfonamide.

Necropsy on cow 13 was performed one hour after death. Death was due to slow exsanguination from multitudinous capillary hemorrhages. Petechiae were universally distributed over the mucosa of the stomach (abomasum) and the whole intestinal tract, and there was a large amount of free blood in the intestine throughout its length. Subepicardial and subendocardial hemorrhages were very extensive, the left ventricular endocardium being most severely involved. Scattered but widespread hemorrhages were found in various other locations throughout the body. Since cultures of the spleen, liver, and kidney failed to produce any bacterial growth, it may be concluded that the hemorrhages were due to toxic effect of the drug, probably to an injury of the capillary endothelium.

Necropsy on cow 89 was performed nineteen hours after death. The kidneys were large and pale, with congestion shown grossly by radial red streaks corresponding to groups of the venulae rectae renis through the medulla. Numerous, tiny, yellowish crystals were barely visible in the medulla; a smaller number was detected in the cortex. These crystals were doubtless the precipitated sulfonamide and, being water soluble, they were absent from microscopic sections. A noticeable degree of focal lymphocytic infiltration was found microscopically.

The right pectineus muscle was pale and degenerated, but fibrous. An area of gluteal musculature about 15 by 10 by 5 cm. showed similar degeneration but less fibrosis. Sections of spinal cord at the first lumbar segment, sciatic nerve, and median nerve, prepared by Marchi's method, showed extensive myelin degeneration. In the cord, practically all fibers of ascending and descending tracts were involved; in the two nerves, from one to several fibers in each nerve bundle were so affected, up to a maximum of an estimated 30 per cent in some bundles. These degenerations would appear to account amply for the paralysis. Figures 6 and 7 show the myelin degeneration observed in the sciatic and median nerves of cow 89.

DISCUSSION

The so-called drug shock from sulfona-

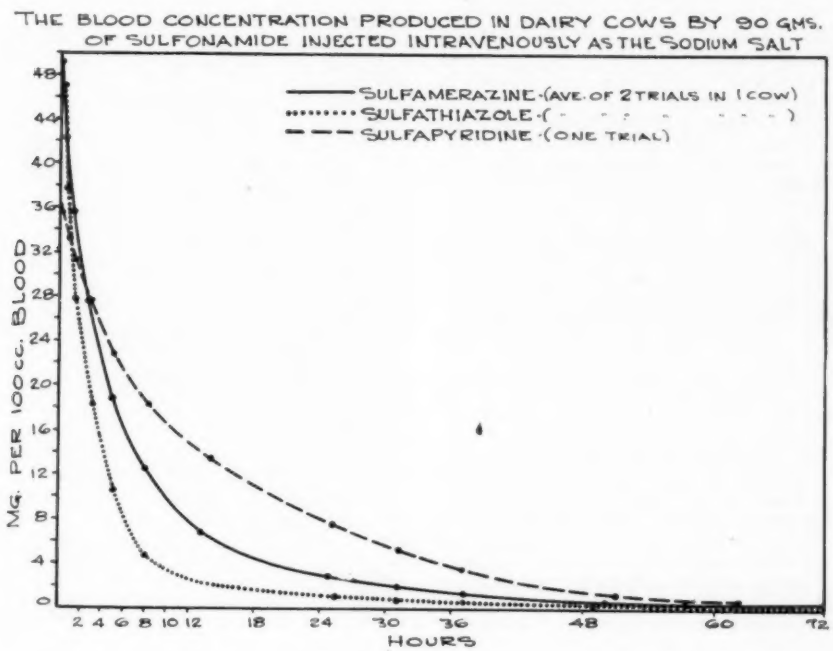

Figure 4

mides injected intravenously has been common in the experience of some veterinary practitioners using this therapy. Most veterinarians have viewed this reaction without alarm because most of such cases recovered.

The clinical signs of distress from drug shock suggested acute involvement of the

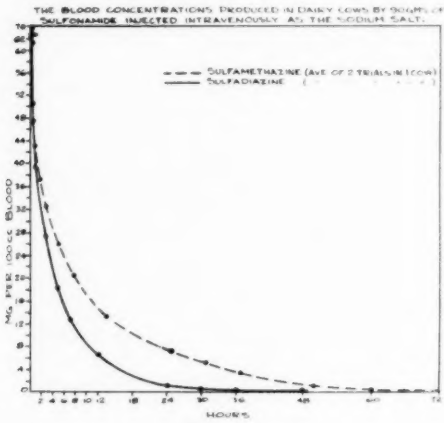
THE BLOOD CONCENTRATIONS PRODUCED IN DAIRY COWS BY 50 GM. OF SULFONAMIDE INJECTED INTRAVENOUSLY AS THE SODIUM SALT.
SULFAMETHAZINE (AVE. OF 2 TRIALS IN 1 COW)
SULFADIAZINE
MG. PER 100 CC. BLOOD
HOURS

Figure 5

central nervous system. Nervous tissue from only 1 cow (89) showing drug shock was examined, but, in this case, extensive nerve degeneration was observed. It seems likely that other cows may also have suffered deranged nervous activity to a lesser degree, inasmuch as the clinical reactions were similar, although varying in intensity and duration. Therefore, a more serious view probably should be taken of drug shock following the intravenous injection of sulfonamides because of the likelihood of acute toxicity to the nervous system. It has seemed that drug shock from the commonly used sulfonamides can be avoided by injecting a solution of the sodium salt slowly and by not using enormous amounts of drug. The above warning seemed justified by the increased tendency of late to administer over larger doses of sulfonamides intravenously and to give them rapidly.

The results of this experiment would indicate that 60-Gm. doses of sulfathiazole, sulfapyridine, sulfadiazine, sulfamerazine, and sulfamethazine were relatively safe to employ when dissolved in 500 cc. of sterile water as the sodium salt and when injected slowly. Sulfamethazine had to be injected more slowly than the above sulfonamides, in this experiment, to avoid shock. The clinical observations suggested that the dairy cow was in less danger of drug shock from sulfathiazole sodium intravenously than any of the others used. This observation was confirmed by the intravenous injection of amounts up to 120 Gm. of sulfathiazole sodium.[1]

Table 4 showed that sulfathiazole left the blood stream faster than the other drugs. This may explain in part why poorer results have been obtained with sulfathiazole in treating foot rot than with sulfapyridine.[1] This fact emphasized the need for oral therapy to supplement that given intravenously in order to maintain the blood concentration of sulfathiazole for longer periods of time. The need of supplementing intravenous therapy with oral therapy has been true of all the sulfonamides to a greater or lesser degree, depending upon the severity of the clinical condition being treated.

Data in table 4 indicated the duration of a sulfonamide in the blood but that did not answer the question as to whether one injection of drug was enough. The answer to that problem was to be found only in the results of clinical trials. The data in table 4 did point out that if the blood-sulfonamide concentration of 2 mg. per 100 cc. of blood was desired during treatment, then a sec-

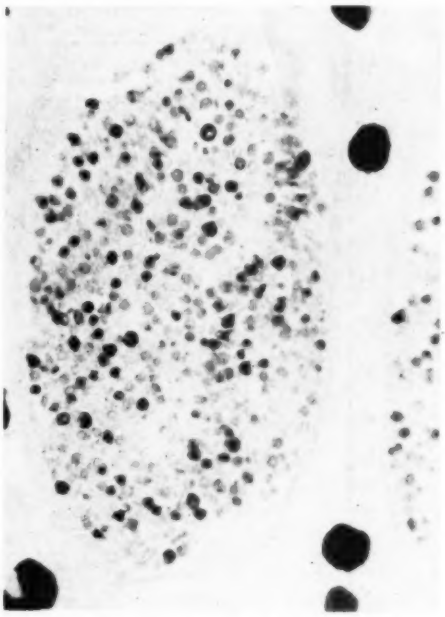

Fig. 6—Myelin degeneration, left sciatic nerve, transverse section. Cow 89. Marchi's technique. x 100.

ond injection of sulfapyridine, for example, was necessary thirty-eight hours after the initial injection of 60 Gm. However, recently reported experimental data indicated that maintenance of a continuously detectable blood-sulfonamide concentration was not necessary for a desirable bacteriostatic action, contrary to previous convictions.

From table 4, it was apparent that sulfapyridine and sulfamethazine left the blood stream more slowly than the other sulfonamides. These compounds would appear to have a longer period of activity than was possessed by the other sulfonamides. However, the question of therapeutic action of these or any sulfonamides can be determined only by experimental clinical trial in the condition under question.

Sulfonamide crystals were found in the kidneys of 2 cows, both of which received sulfaquinoxaline. One of these cows showed myelin degeneration of its nervous tissues. Unquestionably, the dosage of sulfaquinoxaline employed was too large, but, since no guiding information was available, the same dosage was employed as for the other sulfonamides. The occurrence of myelin degeneration in the sciatic nerves and spinal cord has been noted also by Bieter *et al.*[3] in chickens fed various sulfonamides. These lesions were accompanied by a peripheral neuritis, as shown by an ability to move the legs. Peripheral neuritis in a calf dosed with large amounts of sulfathiazole was noted by Jones.[4] The clinical observations on all 3 cows that received sulfaquinoxaline indicated that peripheral neuritis was produced briefly in 2 cows and permanently in 1 cow.

It has been interesting to speculate on the importance of the kidney and liver lesions observed in these animals. For instance, about one half of the kidneys studied microscopically showed a partial loss of cytoplasm from certain tubular cells, much as results from certain metallic poisonings. Some of these facts could be important since sulfonamides are extensively used. However, this experiment was not designed to study the pathology of sulfonamide administration, so no conclusions of that sort are justified.

The death of cow 13 after receiving 60, 60, 90, and 90 Gm. of sulfapyridine was a matter of note. Under clinical conditions, it seemed highly doubtful if a patient ever received as much drug in so short a time. As used in most clinical cases to date, 60 Gm. of sulfapyridine sodium has been injected once. Some patients have been retreated and, obviously, caution was necessary to avoid acute sulfonamide toxicity. The amount of drug, the rapidity of administration intravenously, and the intervals between injection were the most important factors in producing acute sulfonamide toxicity in this experiment.

SUMMARY AND CONCLUSIONS

Sulfamethazine sodium produced a transient "drug shock" in 1 cow after 48 Gm. had been injected intravenously. No shock was noted on subsequent injections.

Sulfaquinoxaline sodium produced severe drug shock in 3 cows after 60 Gm. were injected. One cow did not recover. A later injection of 48 Gm. again caused drug shock.

After the fourth injection of sulfapyridine sodium, 1 cow died, showing failure of the normal blood-clotting mechanism and a progressive decrease in red blood cells and hemoglobin.

Fig. 7—Myelin degeneration, right median nerve, longitudinal section. Cow 89. Marchi's technique. x 100.

No significant variations in body temperature were noted following the administration of the drugs.

Only about 76 per cent of the total amount of sulfaquinoxaline present in a sample of ox blood is accounted for by the standard method of Bratton and Marshall for sulfonamide analysis.

Sulfaquinoxaline temporarily prolonged

the blood-clotting time by two times or more of normal.

Blood-urea nitrogen analyses did not reveal levels widely divergent from the normal range, during and after the administration of sulfonamides intravenously.

After 60-Gm. doses, the drugs disappeared from the blood stream in the following order: sulfathiazole, sulfaquinoxaline, sulfadiazine, sulfamerazine, sulfapyridine, and sulfamethazine.

After 90-Gm. doses, the order of disappearance was the same except that sulfapyridine lasted as long as did sulfamethazine.

After injecting 60-Gm. doses of the above sulfonamides intravenously, concentrations of more than 5 mg. per 100 cc. of blood were maintained for five to twenty-nine hours, respectively, and concentrations of more than 2 mg. for eight to forty-four hours, respectively.

After injecting 90-Gm. doses of the above sulfonamides intravenously, concentrations of more than 5 mg. per 100 cc. of blood were maintained for eight to thirty-three hours, respectively, and concentrations of more than 2 mg. for fifteen to forty-six hours, respectively.

Precipitated sulfonamide crystals were found in the kidneys of 2 of the 3 cows that received sulfaquinoxaline.

Microscopically, kidneys from all the cows showed acute passive congestion of the medullary venules; one third showed albuminous casts; and one half showed partial loss of the cytoplasm of the tubular epithelium.

Microscopically, all the liver sections showed cloudy swelling and albuminous degeneration, sometimes with an advancing necrosis of hepatic epithelium and sometimes accompanied by intracellular fat.

Death of cow 13 appeared due to some toxic effect of sulfapyridine upon the normal blood-clotting mechanism and perhaps upon the fragility of the capillaries.

Death of cow 89, which received 60 Gm. of sulfaquinoxaline, probably resulted from the extensive myelin degeneration of the tissues of the central nervous system, as shown by the first lumbar section of the spinal cord. Certainly, the paralysis of the legs could be explained by the myelin degeneration observed in the sciatic and median nerves.

References

[1]Forman, C. R., Burch, J. E., Dee, C. E., Kelley, L., Mouw, J. E., Teigland, M. B., and Yarborough, J. H.: Use of Sodium Sulfonamides as Single Injection Specific Treatment in Foot Rot. J.A.V.M.A., *111*, (1947):208-214.

[2]Bratton, A. C., and Marshall, E. K.: A New Coupling Component for Sulfanilamide Determination. J. Biol. Chem., *128*, (1949):537-540.

[3]Bieter, R. N., Baker, A. B., Beaton, J. G., Shaffer, J. M., Seery, T. M., and Orr, B. A.: Nervous Injury Produced by Sulfanilamide and Some of Its Derivatives. J.A.M.A., *116*, (1941):2231-2236.

[4]Jones, L. M.: The Chemotherapy of Calf Pneumonia. II. The Use of Sulfathiazole, Sulfapyridine, and Sulfadiazine in the Treatment of Calf Pneumonia. Am. J. Vet. Res., *8*, (1947):14-28.

Tissue-Respiration-Stimulating and Thromboplastic Activity of Bovine Lung Extracts

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WHILE connected with Medical Research of Chemical Warfare Service during the war period 1942-1944, one of us (R.W.D.) began some studies on the biologic activity of certain crude aqueous lung extracts.

Because of the lack of time and certain laboratory equipment, these observations were limited to the study of the thromboplastic and wound-healing activities of lung extracts from various species, compared to tissue extracts of brain, heart, spleen, liver, and striated and smooth muscle taken from several species. The first part of this report will be devoted to the original army work. The latter part will include confirming and additional work done at the State College of Washington, Pullman, Wash.

METHODS AND PROCEDURE

Crude aqueous lung extracts were made from cattle, goat, pig, and sheep lungs. Bovine lung extracts seemed to be just as active as lung extracts from any of the other species tried. Since these lungs were available, most of the observations were made using bovine lung extract. In some of the trials on clotting and experimental surgery, lung extract was desiccated under vacuum at room temperature. In these experiments, the lung extract seemed to be just as active in the powdered as in the liquid form.

Lung extract was made as follows: Bovine lungs were obtained from abattoirs in as clean a condition as possible. If aspirated ingesta were noticed in the respiratory tree, the lungs were discarded, since contaminating material greatly impaired the keeping qualities of the extract. As nearly as possible, parenchymatous tissue, only, was used. The lungs were ground in a household meat grinder; they were then macerated in a Waring blender, with enough distilled water or physiologic saline to make a thin emulsion. This material was then passed through a colloid mill so that more complete tissue and cell breakdown could be effected. At first, a rather crude mill was used. Later, an Eppenbach Q V-6 colloid mill was used more effectively. The finely emulsified material was then centrifuged, using a Sharples supercentrifuge, at speeds varying between 25,000 and 50,000 r.p.m. The final liquid material was kept at ordinary household refrigerator temperature.

Several of the sulfonamides and certain antibiotics were used as preservatives, with some success; they apparently did not reduce the blood-clotting and tissue-respiration-stimulating activity of the extracts. Due to the availability of lungs and the ease of making fresh extract, fresh and unpreserved extract was used in most of the experiments.

In this report, lung extract will be referred to as LE, and it will be assumed to be of bovine lung origin. The uptake of gaseous oxygen by tissue *in vitro* will be referred to as "respiration" or "tissue respiration."

The clot-stimulating activity was studied from two aspects, the clotting time and the tensile strength of the clots.

In the characterization studies of lung extract, it was not known how many factors were responsible for the activities under study, so that it was thought advisable to carry on the blood-clotting experiments in conjunction with the other studies.

Lung extract decreased the clotting time of fresh horse blood from eleven to fifteen minutes to fifteen to twenty seconds. It was effective in liquid and powdered forms. The quantities required to clot 2 cc. of horse blood were very small. As little as 0.0001 cc. of LE appreciably decreased the clotting time of fresh horse blood.

Lung extract, in 0.1-, 0.2-, and 0.3-cc. amounts, was added to 2-cc. amounts of fresh horse blood. Clotting occurred in fifteen to eighteen seconds. One-tenth, 0.2, and 0.3 cc. of serum from these series of clots were used to stimulate clotting in the next series of 2-cc. amounts of horse blood. The clotting time of the tenth series of fresh horse blood was still about half that of the controls.

The tensile strength of clots was reduced, according to the testing methods employed. Sheep, ox, and horse blood were clotted *in vitro*, with and without the addition of bovine lung extract. The force necessary to break the clot was measured by a method similar to the one described by Charney, Williamson, and Bernhart[1] and used in measuring the tensile strength of wounds. Although experiments were limited, there was some indication in the early work that clots were strengthened when LE was placed in contact with experimental

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At present, Dr. Dougherty is at New York State Veterinary College, Cornell University, Ithaca; and Dr. Conner is at the School of Veterinary Medicine, University of Minnesota, St. Paul.

The authors gratefully acknowledge the help given by Drs. E. C. McCulloch and D. R. Cordy of the College of Veterinary Medicine, State College of Washington, Pullman.

wounds. This is an important point and needs further investigation.

In the early army work, LE very definitely promoted rapid healing in experimentally induced wounds and skin grafts. In goats, there

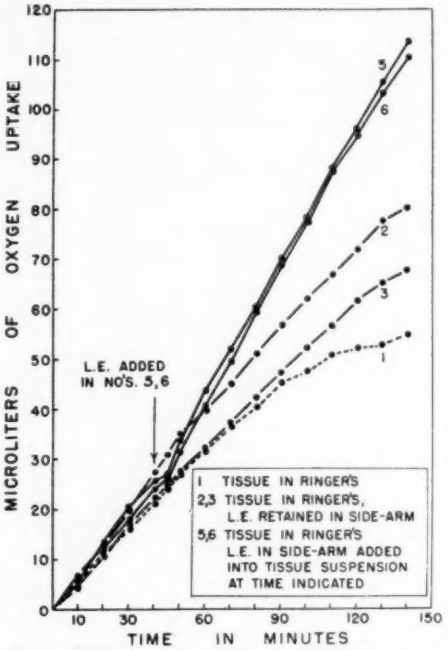
MICROLITERS OF OXYGEN UPTAKE
TIME IN MINUTES
L.E. ADDED IN NO'S. 5,6
1 TISSUE IN RINGER'S
2,3 TISSUE IN RINGER'S, L.E. RETAINED IN SIDE-ARM
5,6 TISSUE IN RINGER'S L.E. IN SIDE-ARM ADDED INTO TISSUE SUSPENSION AT TIME INDICATED

Chart 1—Effect of lung extract on rabbit-liver tissue O_2 uptake.

was a definite decrease in the healing time of experimental wounds, when LE was applied to the wounds immediately after they were made. Whether this was due to a definite stimulation of cells surrounding the wounds, by increasing the rate of proliferation, or whether it was due to the mechanical effect of better adhesion of the lips of the wounds is a question of considerable importance.

In order to further study the nature of LE and its possible mechanism of action, a series of experiments were performed using Warburg's "direct method," *i.e.*, the determination of respiration (oxygen uptake) by absorbing the liberated carbon dioxide in alkali.[2] Liver tissue, taken from freshly killed rabbits, was sliced into approximately 200-mg. pieces, using the method of Deutsch.[3] Wet weights of the slices were obtained on a torsion balance. The tissue slices were placed in the reaction flasks, containing 3.0 cc. of Krebs-Ringer's phosphate solution. Lung extract, in amounts indicated in the graphs, was placed in the side-arms, and 0.3 cc. of KOH (10%) was added to the center cup. The temperature of the water bath in all the experiments was 38 C. Vessels were allowed to equilibrate with shaking for fifteen minutes, the oscillatory rate being 100, 2-cm. strokes per minute, before readings were begun. The reactions were allowed to continue for at least ninety minutes after the side-arm contents were added.

All charts included in this report are typical of a significant number of trials.

RESULTS

1) In all experiments performed, it was found that fresh, untreated LE had a marked stimulating effect on the respiration rate of the excised rabbit liver tissue. The increase in oxygen uptake ranged from 1.2 to 2.0 times that of the controls. Results shown in chart 1 are typical. Controls, run with LE alone, showed the oxygen uptake by the extract to be negligible.

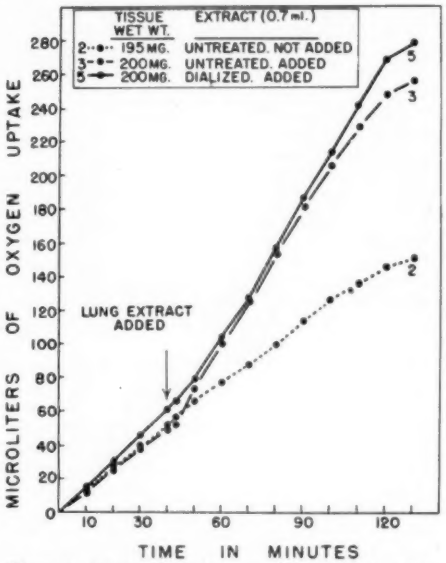
TISSUE WET WT.
EXTRACT (0.7 ml.)
2 195 MG. UNTREATED. NOT ADDED
3 200 MG. UNTREATED. ADDED
5 200 MG. DIALIZED. ADDED
MICROLITERS OF OXYGEN UPTAKE
TIME IN MINUTES
LUNG EXTRACT ADDED

Chart 2—Effect of dialysis on lung extract action.

2) Lung extract was dialyzed against distilled water, using a cellophane membrane. Experiments run, using the dialysate, showed that the stimulating factor was retained in the original solution. It was not dialyzable through the membranes used, as shown in chart 2.

3) Changes in pH of LE, using either mild acid or alkali, reduced the stimulating effect of the extract on liver tissue. The unaltered LE showed the greatest stimulating effect. Lung extract, altered to pH of 5.0 and 8.0, actually had an inhibitory effect upon the tissue respiration, as seen in chart 3.

4) Seitz filtration of LE reduced the stimulating action slightly. Even the fil-

tered material, however, increased respiration of the excised tissue (*see* chart 4).

5) Effects of heat and cold on the tissue-respiration-stimulating factor were studied. Lung extract, heated over 45 C. for more than fifteen minutes, failed to display any stimulating action. Storage of LE, at 5 C. for thirty to sixty days, and freezing at –20 C. for eighteen hours, had no appreciable effect upon its activity.

7) Lung extract was found to be markedly superior to either bovine spleen extract or rabbit spleen extract in inducing increased respiration in excised rabbit liver tissue.

8) The possibility of accelerating the respiration of excised rabbit tissue with bovine serum was considered. A study of the comparative effect of LE and of bovine serum revealed no stimulating action with

VESSEL	TISSUE WET WT.	pH OF L.E. (0.7 ml.)	AV. 10 MIN. UPTAKE BEFORE ADDING L.E.		AV. 10 MIN. UPTAKE AFTER ADDING L.E.	
			TOTAL μl.	μl/MG.	TOTAL μl.	μl /MG.
2	205 MG	6.7*	9.48	.046	11.11	.054
3	200	6.0	8.65	.043	9.10	.046
4	215	5.0	10.56	.049	9.45	.043
5	230	8.0	12.18	.052	8.62	.037
7	200	7.0	8.81	.044	9.24	.046

* UNALTERED, ORIGINAL L.E.

Chart 3—Effect of pH alteration in lung extract on its action.

6) Removal of the factor with half-saturated and saturated solutions of ammonium sulfate was attempted. Lung extract, treated with saturated salt solution, showed no stimulating activity; LE treated with half-saturated salt solution showed a great reduction in its ability to stimulate tissue respiration.

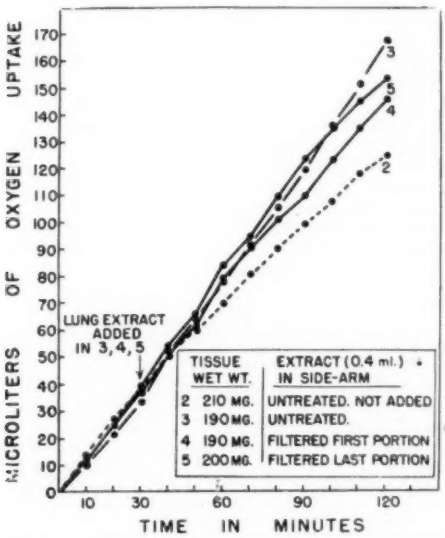
MICROLITERS OF OXYGEN UPTAKE
TIME IN MINUTES
LUNG EXTRACT ADDED IN 3, 4, 5
TISSUE WET WT.
EXTRACT (0.4 ml.) IN SIDE-ARM
2 210 MG. UNTREATED. NOT ADDED
3 190 MG. UNTREATED.
4 190 MG. FILTERED FIRST PORTION
5 200 MG. FILTERED LAST PORTION

Chart 4—Effect of Seitz filtration on lung extract action.

the latter, while LE was found to double oxygen uptake in ninety minutes, as shown in chart 5.

Dr. C. F. Sprague, biochemist in the Department of Physiology, New York State Veterinary College, Cornell University, Ithaca, N. Y., using Warburg's direct manometric method and with a limited number of trials, has confirmed the tissue-respiration experiments done at the State College of Washington and has further found that a liver homogenate is more active than liver slices, when exposed to LE.

The following experiments were performed to determine if the clot-stimulating characteristics of LE were due entirely to its contained thromboplastic substance.

For this study, 6 young range ewes, that had not had access to sweet clover, were used. Before administering synthetic dicoumarin (dicumarol), clotting times were determined for all 6 sheep on each of two successive days. These clotting times, and those taken throughout the study, were determined by the capillary-tube method.

Four ewes were used for dicoumarin administration; the remaining 2 were controls and received no dicoumarin. Those receiving the drug were given identical oral doses on the same day.

Five days after the beginning of the experiment, it was decided that prothrombin-clotting times should be determined. This procedure was then done daily on all 6 sheep and, to a large extent, replaced the taking

of clotting times, although, on some days, both the clotting times and prothrombin-clotting times were determined.

The procedure for determining the prothrombin-clotting time was that described

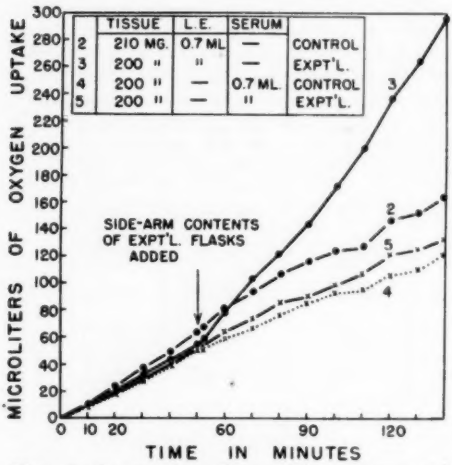
TISSUE
L.E.
SERUM
2 210 MG. 0.7 ML — CONTROL
3 200 " " — EXPT'L.
4 200 " — 0.7 ML. CONTROL
5 200 " — " EXPT'L.
SIDE-ARM CONTENTS OF EXPT'L. FLASKS ADDED
MICROLITERS OF OXYGEN UPTAKE
TIME IN MINUTES
0 10 20 60 90 120
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
2
3
4
5

Chart 5—Comparative effects of lung extract and bovine serum.

by Quick,[4] with the exception of the source of thromboplastic substance, which in this case was LE. The same lot of thromboplastic substance was used in all of the determinations of the prothrombin-clotting times made in this study.

When a total of 3,700 mg. of dicoumarin had been given (9 days' time) to each of the 4 treated sheep, administration of the drug was stopped, because 1 ewe was becoming moribund and the other 3 were showing symptoms of dicoumarin poisoning. At this point, the prothrombin-clotting times of the 4 treated ewes were over ten minutes and the clotting times varied from fifteen and a half to twenty minutes. Determinations of prothrombin-clotting time and a few clotting times were continued for eight days after cessation of dicoumarin administration; after which time, all prothrombin-clotting times were within the time range determined on the 2 control ewes. As soon as four days after dicoumarin administration was stopped, the prothrombin-clotting times were within the established normal range. Normal prothrombin-clotting times varied between thirteen and twenty-five seconds.

Blood from the dicoumarinized sheep would not clot when lung extract was added; however, it would clot readily when thrombin alone was added. This indicated that lung extract is rich in thromboplastic substance, but contains very little prothrombin and/or thrombin.

It has been shown[6] that thromboplastin, prepared from brains of rabbits poisoned with dicoumarin, was less active than that obtained from normal animals. The diminution of thromboplastic activity, however, is not particularly marked.

Numerous attempts were made to determine whether two factors were present or if thromboplastic substance could also be responsible for increased *in vitro* tissue respiration. No positive information has been gained. It is known definitely that a crude aqueous extract of bovine lung has two properties: (1) It greatly accelerates clotting of normal blood; (2) *in vitro*, oxygen uptake of excised rabbit liver is markedly increased.

SUMMARY

1) A method for making a crude lung extract has been described. Two properties of the extract have been studied.

2) Lung extract accelerates blood clotting: 0.0001 cc. will accelerate the clotting of 2 cc. of freshly drawn blood.

3) The extract is active in the liquid and desiccated forms.

4) Lung extract lessens the healing time of experimental wounds when applied to the fresh bleeding surfaces.

5) The clot-stimulating properties are apparently due to thromboplastic substance. It will not clot blood from sheep with low prothrombin level (animals having been fed toxic amounts of dicoumarin).

6) *In vitro* studies show a marked increase in oxygen uptake of excised rabbit liver when lung extract is added, the principal increase occurring within ninety minutes. Bovine serum had no stimulating effect on excised rabbit liver.

7) The factor or factors which accelerate tissue respiration and the blood clotting are thermolabile, being affected by increased temperatures; are inhibited by changes in pH; are not dialyzable through cellophane membranes; are not affected by low temperatures; and are salted out with half-saturated and saturated solutions of ammonium sulfate.

References

[1]Charney, Jesse, Williamson, Martin B., and Bernhart, Finn W.: An Apparatus for the Determination of the Tensile Strength of Wounds. Science, *105*, (1947):396.

[2]Umbreit, W. M., Burris, R. H., and Stauffer, J. E.: Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Burgess Publishing Co., Minneapolis, Minn., 3rd printing, January, 1947.

[3]Deutsch, W.: An Improvement of Warburg's Method for Cutting Tissue Slices for Respiratory Experiments. J. Physiol., *87*, (1936):56.

[4]Quick, A. J.: On the Quantitative Estimation of Prothrombin. Am. J. Clin. Path., *15*, (1945):560-566.

[5]Stefanini, M., and Blanchaer, M. C.: Action of 3,3' Methylenebis (4-Hydroxycoumarin) (Dicumarol) on Thromboplastic Activity of Rabbit Brain. Proc. Soc. Exptl. Biol. and Med., *64*, (1947): 47-50.

Studies on the Subgross Anatomy of the Bovine Liver

I. The Distribution of the Blood Vessels and Bile Ducts as Revealed by the Vinylite-Corrosion Technique

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SOLIDIFYING injection masses with subsequent corrosion were used in anatomic studies previous to 1670.[1] Beeswax, paraffin, celloidin, metals of low melting points, and, in recent times, latex and plastics have been used by various workers to demonstrate blood · vessels and other hollow structures.[1,2,3,4,5] These methods serve to demonstrate the "subgross"* anatomy of an organ. In an organ such as the liver, in which the accompanying vascular network is a major part of its anatomy, a study of the distribution of the blood vessels assists in obtaining a clearer understanding of its internal arrangement. The purpose of this report is to present observations on the subgross anatomy of the bovine liver.

METHOD OF PROCEDURE

The livers used were obtained from a local packing plant, and, in most instances, preparation of a cast was begun less than six hours after the death of the animal. The caudate lobe of the liver was used because it has but one major branch of the hepatic vein, the hepatic portal vein, the hepatic artery, and the bile duct entering its substance. This fact made the caudate lobe particularly useful, and, only when it was necessary to relate the structures observed in casts of the caudate lobe with the structures of the liver as a whole, was the entire liver injected.

The general technique for the preparation of vinylite-corrosion casts, as described by Narat, Loef, and Narat,[1] Puckett and Neuman,[3] and Cottral,[4] was applied with but few modifications. First, the blood was removed from each vascular tree by the injection of tap water until the return flow of water was clear. Then, air was injected to force the water from the vessels. Next, acetone was forced into the vessels, since 12.5 per cent solutions of vinylite in acetone were to be used as injection mediums.[4] Air was again used to free the vessels of liquid. Different colored vinylite solutions were then injected into each vascular system, and the preparation was allowed to stand until the vinylite had solidified. The specimen was immersed in crude concentrated hydrochloric acid until the tissues had been corroded from the cast. The cast was then rinsed in a slowly moving stream of water for twenty-four hours, dried, trimmed, and mounted.

The pressure needed to satisfactorily demonstrate the sinusoids of the liver causes serious distortion of the organ; hence, in none of the casts upon which this study is based, were the sinusoids injected. An incompletely injected cast may reveal features which would be

Fig. 1—The distribution of the vessels and bile ducts in the liver of a calf, as seen from the visceral surface. A, hepatic vein; B, hepatic portal vein; C, hepatic artery; D, bile duct; E, primary branch of hepatic portal vein leading into the caudate lobe. This illustration shows only the general distribution of the largest vessels, and does not adequately portray the complex arrangement seen in the corrosion models. 0.29 life size.

From the Department of Veterinary Science, University of California, Berkeley.

*The term "subgross anatomy" has been designed to cover the information available through a group of techniques which reveal the correlation between gross and microscopic anatomy. Subgross anatomy is best revealed at magnifications of 6 to 40 diameters.

The above study was partially supported by funds presented by Swift and Company.

missed in a more completely injected one, since the latter presents a maze of vessels of such complexity that a careful study of their arrangement is difficult.

The shrinkage which followed the use of vinylite afforded no serious handicap in arriving at an interpretation of the distribution of

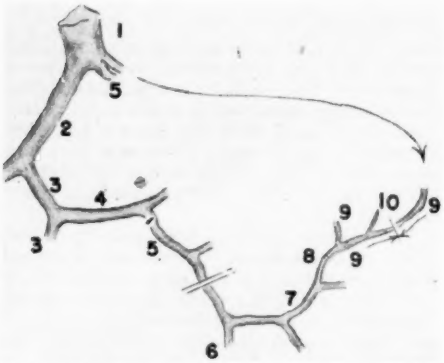

Fig. 2—The distribution of the hepatic portal vein within the caudate lobe of the liver of an adult animal. The vessel diminishes in size from the primary incoming vessel (1) to the interlobular vessels (10).

the vessels. The parenchyma of the liver limits the shrinkage to a contraction of the individual vessels in a transverse plane rather than a longitudinal one. However, a proper period of time must be allowed for the plastic to harden.

RESULTS

Figure 1 is a simplified diagram of the distribution of the major branches of the hepatic vein (A), hepatic portal vein (B), hepatic artery (C), and bile ducts (D) within the liver. The drawing was made from the visceral surface of a corrosion model of the liver of a calf and shows the branches of the hepatic artery and portal vein coursing toward the periphery of the organ. The branches of the hepatic vein are shown in their paths away from the terminal regions of the liver. The hepatic veins open into the vena cava close to the left border. The close spacial relationship of the branches of the hepatic portal vein, hepatic artery, and bile ducts is illustrated by this drawing. These three components of the liver are found in the portal canals. It will be noted that one of the primary branches of the incoming hepatic portal vein leads into the caudate lobe (E). Dissection of fresh specimens of adult livers disclosed this condition to exist in the adult, as well as the calf. The primary branch of the portal vein, entering the caudate lobe, corresponds to the vessel labeled "1" in figure 2.

The hepatic portal vein was traced from its point of entrance into a caudate lobe of an adult animal to its terminal branches. By comparing the diameters of succeeding segments of the vein, approximately ten gradations in size were noted. These are labeled "1" through "10" in figure 2. It must be stated that the divisions are quite arbitrary, based only upon their apparent diminution of size. The exact number of graded sizes varies from specimen to specimen and appears to be determined by the size of the liver. Only two constant points of orientation are available in the consideration of the distribution of the hepatic portal vein. These are, point "1" of the illustration, which represents a primary branch from the hepatic portal vein, and the terminal branch, "10." The latter vessel is an interlobular radical of the portal vein (A of fig. 4).

The distribution of the hepatic artery closely follows the pattern set by the portal vein. The major difference is that, in the larger portal canals, many branches of the artery may accompany a single branch of the portal vein.

An important feature disclosed in chart-

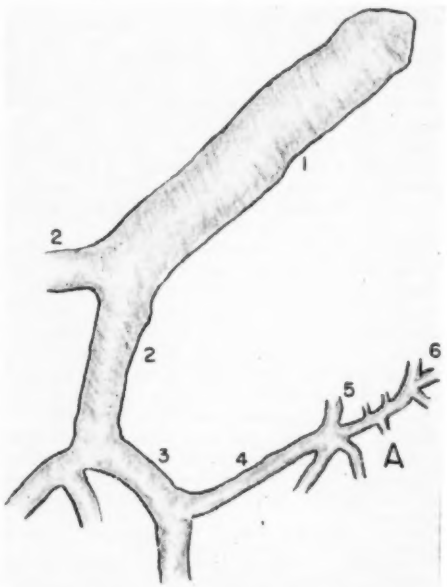

Fig. 3—The distribution of the hepatic vein within the caudate lobe. A, site of origin of three small vessels, each of which is smaller than the succeeding segment (6).

ing the course of the hepatic portal vein is the existence of "portal circuits." A portal circuit (demonstrated in fig. 2) consists of an anastomosis of one of the finer branches of the portal vein with a vessel of corresponding size having its origin from another portion of the portal vein. Thus, in the illustration, it may be seen that the major vessel diminished in size to point "9," where it then became continuous with a portion of the portal vein which previously had branched from point "1." Physiologically, this anatomic feature means that in the region of the anastomosis, the parenchyma may be supplied by two portions of the portal circulation.

In comparing the hepatic vein with the portal vein, the most striking difference is the greater diameter of the former. In the cases of both the hepatic vein and the portal vein, the diminution in size of a given primary branch (1 in fig. 2 and 3) is uniform and progressive for only the major continuing portion of the branch. That is, in figures 2 and 3, the major vein continues through the various steps of diminishing size already discussed, but any one of the segments may give rise to radicals which are much smaller than the succeeding segment. Thus, at "A" in figure 3, three distinct sizes of veins branched from a fifth order vein. All three of these branches were smaller than the succeeding order (6). This feature is particularly striking in the case of the hepatic vein, in which central veins may be seen branching at right angles from the largest branches. Mall[2] has found a similar condition to exist in the human liver. This feature is not surprising when it is considered that, throughout their course, the veins are embedded in the tissue which they supply. The necessity for small vessels to nourish the adjacent tissue is met by supplying small vessels directly from the large ones.

Figure 4 is a drawing of the veins of an individual lobule. Two interlobular portal veins are seen at the periphery of the lobule (A), and one central vein is shown at the center of the lobule (B). Branches of the interlobular veins are seen coursing toward adjacent lobules (C). The vessels related to the individual lobule were identified by determining the average diameter of the lobule from frozen pieces of liver. Since the average diameter was found to be approximately 1 mm., small portal veins which were 1 mm. apart were then located in the corrosion specimens. It is interesting to note that the diameter of the lobule of the bovine and human livers are approximately the same.[6] The bifid nature of the central vein, shown in figure 4, has been described in the human liver.[6]

A corrosion cast of the entire liver demonstrated a noteworthy feature of the relationship between the components of the portal canals. A specimen in which the biliary system has been injected will show a vast plexus of small arteries surrounding the bile duct (*see* fig. 5). These arteries run parallel to, or diagonally around, the duct and arise from branches of the adjacent hepatic artery. A study of this plexus discloses that, in the course of the hepatic artery, branches arise from the main trunk and are directed toward the bile duct (fig. 5 and 6, C). These branches divide into ascending and descending components within a few millimeters of their origin. The

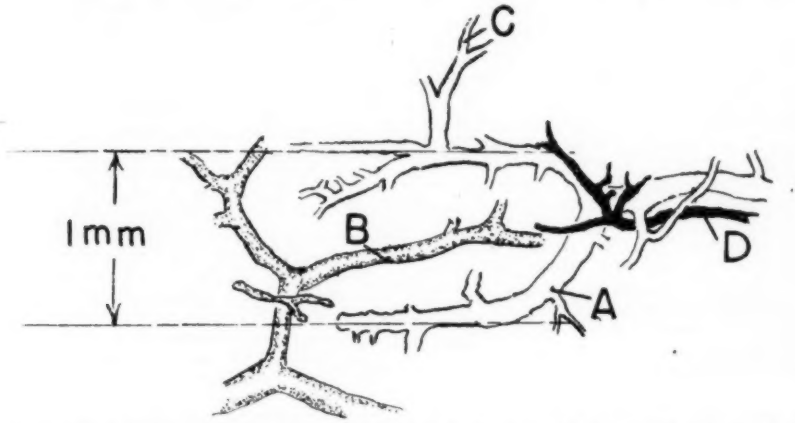

Fig. 4—Detail of the vessels related to the individual liver lobule. A, interlobular portal vein; B, central vein; C, radical of interlobular portal vein directed toward an adjacent lobule; D, small bile duct. x 25.

descending components course down the duct and anastomose with ascending components of a similar artery (fig. 6, E) arising from the main arterial trunk. The ascending branches course up the duct to unite with descending branches from another similar artery of the main trunk (fig. 6, A). A netlike plexus is formed by the longitudinal vessels just described and by

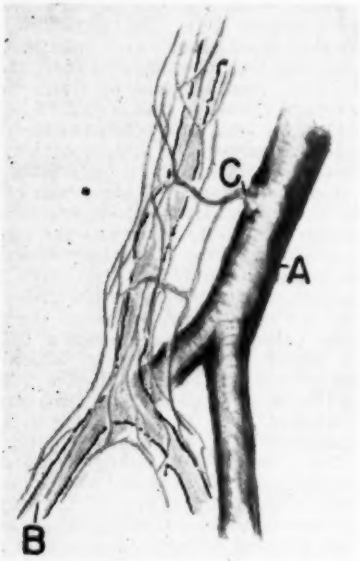

Fig. 5—Detail of an arterial plexus about a large bile duct. A, branch of hepatic artery; B, bile duct; C, small artery from which the periductal plexus arises. x 12.

lateral anastomoses between them (fig. 6, D). It should be stressed that the vessels illustrated in figures 5 and 6 are small arteries; they are not capillaries.

The arterial plexus just described is best studied in portions of the liver in which but one artery comprises the arterial component of the portal canal. At the origins of the hepatic canals and throughout the distance represented by the first segment of the primary branches of the portal vein, the hepatic artery, and the bile duct, the arterial arrangement is so dense that the plexus cannot be clearly seen. The density of the plexus about the bile duct gradually diminishes as the vessels of the portal canals progress peripherally.

DISCUSSION

In presenting the structure of the portal circuit, it has been mentioned that the tissue in the vicinity of the anastomosis which completes a circuit has a potential supply of portal blood from two sources. Much work has been done on the subject of a possible connection between arterial and venous blood supplies.[6] Little attention has been paid to the possible existence of connections between the portions of an individual vascular system. Portal circuits may be important in various pathologic states of the bovine liver. In a subsequent report, dealing with telangiectasis of the bovine liver, it will be shown that lesions are occasionally demonstrated within portal circuits. Two portions of the portal circulation contribute to the formation of such lesions.

The periductal arterial plexuses may have their prime function in supplying nutrition to the large bile ducts. Cameron and Mayes[7] have demonstrated small arteries branching from the intrahepatic arteries of the rabbit's liver. These break up into capillary plexuses which are distributed to the bile ducts and portal veins. Although the bile ducts of the rabbit's liver are supplied by arterial capillary plexuses, the ducts apparently are not dependent upon the capillaries for their maintenance. Ligation of the hepatic artery causes widespread necrosis of the liver tissue, but the bile duct remains apparently unharmed. It is possible that the plexuses of small arteries seen about the bile ducts of the bovine liver are analogous to the capillary plexuses described in the liver of the rabbit. It is reasonable to suppose that, since the major bile ducts of the bovine liver are larger than those of the rabbit, they may require a more complex arterial network.

It is possible that the complex capillary networks associated with the arterial plexuses may function in changing the composition of the bile as it passes through the ducts.

Knisely[8] states, ". . . the functioning of the vascular bed of any organ, . . . (is) closely integrated with the other structures and functions of the organ." ". . . capillaries of each organ are as much a part of that organ as are any of the organ's constituent parts." There is probably no organ in which these statements are more applicable than the liver. A large proportion of the total weight of the liver is blood. The vessels have a marked influence on the development, the growth, and the regeneration, as well as the physiologic activity, of the liver. An appreciation of the anatomic bases of these considerations can best be obtained from the realm of subgross anatomy. For example, the periductal plexuses and the portal circuits described in this paper exist in three dimensional patterns in space. Their arrangement cannot be appre-

ciated in the gross specimen. Two dimensional histologic preparations, unless used to produce serial-section reconstructions, do not adequately demonstrate their third dimensional aspect. The field of subgross anatomy has been relatively untouched. It is felt that its importance has not been properly appreciated and, there is little doubt, that within this realm of anatomy lie many facts important to the total understanding of anatomic arrangement and physiologic and pathologic function.

The technique described in this paper may provide a means by which the blood volume of a given organ can be estimated. This may be accomplished by measuring the amount of vinylite required to inject the vessels of an organ. The procedure is currently under investigation. In the case of the liver, it appears to give a minimum value for the extralobular blood volume of any one or all of the vascular systems of the organ.

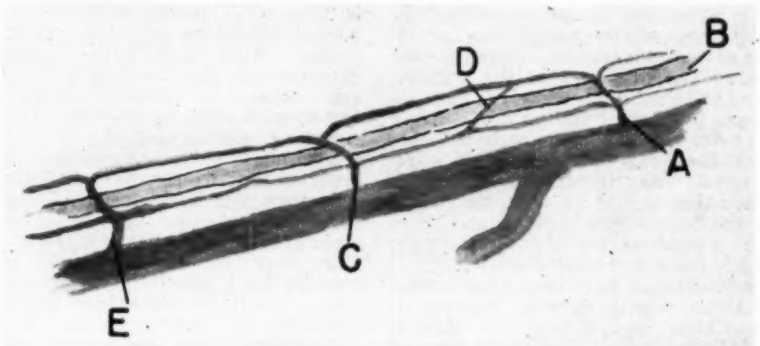

Fig. 6—Schematic illustration of the arrangement of a periductal plexus. A, small artery from the hepatic artery; B, bile duct; C, small artery from the hepatic artery; D, lateral anastomosis of small longitudinal periductal arteries; E, small artery from hepatic artery.

SUMMARY

1) The results of a study of the "subgross" anatomy of the bovine liver are presented. It is in the realm of subgross anatomy that organization of an organ may be best understood. Vinylite-corrosion casts of the vascular systems have been used to study the bovine liver.

2) The existence and the arrangement of "portal circuits" is described. The circuit is formed by the anastomosis of two fine portal branches, each of which originates from a moderately sized portal radical.

3) Extensive plexuses of small arteries surround the large bile ducts. The structure of the plexuses is presented.

It is suggested that the techniques employed in the demonstration of subgross anatomy have many potentialities in presenting, in three dimensions, details of internal arrangement necessary for a more complete understanding of structure and functions of an organ. It is further suggested that these techniques may be used to estimate the blood volume of an organ.

References

[1]Narat, Joseph K., Loef, John A., and Narat, Mitzi: On the Preparation of Multicolored Corrosion Specimens. Anat. Rec., *64*, (1936):155-160.

[2]Mall, Franklin P.: A Study of the Structural Unit of the Liver. Am. J. Anat., *75*, (1906):227-308.

[3]Puckett, W. D., and Neuman, C. P.: Vinylite Resin in the Preparation of Corrosions of Anatomical Specimens. Anat. Rec., *78*, (1940):105-111.

[4]Cottral, G. E.: Vinylite Resin Corrosion Preparations in the Study of Avian Leucosis Complex. Am. J. Vet. Res., *3*, (1942):227-234.

[5]Bechtel, Ernest H., and McLeod, W. M.: Latex in the Preparation of Corrosions of Mammary Glands. Am. J. Vet. Res., *6*, (1945):17-20.

[6]Lichman, S. S.: Diseases of the Liver, Gallbladder and Bile Ducts. Lea and Febiger, Philadelphia, Pa., (1942):16.

[7]Cameron, G. R., and Mayes, B. T.: Ligation of the Hepatic Artery. J. Path. and Bact., *33*, (1930):799-831.

[8]Knisely, Melvin H.: The Histophysiology of Peripheral Vascular Beds. In "Blood, Heart and Circulation." Science Press, Washington, D. C., (1940):303-307.

Brucellosis Therapy: Studies on the Effect of Streptomycin and Sulfadiazine in Experimental Brucellosis in Guinea Pigs

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THERAPY OF brucellosis in domestic animals has long remained in an unsatisfactory state, although treatment of human brucellosis has advanced markedly within the past two years. The discovery of the sulfonamides and the antibiotics has given new impetus to the problem of finding and utilizing a drug or drugs which, alone or in coöperation with the body defenses, would eliminate Brucella from the tissues. The drug or combination should be nontoxic to the host at the concentrations used, and there should be a good margin of safety between therapeutic doses and toxic doses.

The sulfonamides have been used in brucellosis therapy with varying degrees of success. Chinn[3] and Wilson and Maier[28] treated infected guinea pigs at the time of infection with sulfanilamide and sulfapyridine, respectively, and found sterile spleens and livers at autopsy. Dumaresq[7] treated guinea pigs with massive doses of sulfanilamide and reported a retardation of the disease. Comparable treatment in cows produced no cures.

In therapy of cows, Schuhardt, Rich, and Beal[24] reported that sulfadiazine produced no cures. Only mediocre results were obtained by Live, Stubbs, and Gardiner[18,19] after treating infected cows with sulfathiazole and sulfapyridine. The conflicting reports concerning sulfonamide therapy in human patients are reviewed by Spink *et al.*[26]

Penicillin is not intensely active against Brucella but T'ung[27] found that eight of the 15 strains tested *in vitro* showed some susceptibility to the antibiotic. Berman, Irwin, and Beach[1] treated infected cows with penicillin but no cures were achieved. Bunnell, Hutchings, and Donham[2] infected guinea pigs with *Brucella suis* and treated the animals with penicillin at various levels and at various times after exposure. The course of the disease was not significantly altered.

Although Brucella organisms are very susceptible to the action of streptomycin *in vitro*,[4,11,23] results obtained in experimental infections in guinea pigs and in human cases have been disappointing. Streptomycin was used by Live, Sperling, and Stubbs[20] in treating guinea pigs infected with *Brucella abortus*. The conclusion was reached that the drug protected the animals. Kelly and Henley,[14] however, reported that streptomycin is of little value in guinea pigs infected with *Br. suis*. Gilman and LeGrow[10] used streptomycin to treat guinea pigs infected with *Br. abortus*. The drug was administered intraperitoneally for a relatively short time. Under these conditions, streptomycin failed to overcome or prevent guinea-pig brucellosis. Case reports from various sources[9,21] indicated that the majority of human patients was not cured by streptomycin therapy.

Recent data by Cotton and Swope[6] indicated that the sodium salt of para-aminobenzoic acid (NaPAB), given subcutaneously every four hours for twenty-one days at a level of 3 Gm. per kilogram, produced complete tissue sterilization if the treatment were started three days after infection. When therapy was begun two weeks after infection, 80 per cent of the infected animals showed tissue sterilization.

Among those engaged in therapy of infectious diseases, there is a growing tendency to combine two therapeutic agents to produce, in many instances, more complete cures. Kolmer[16] has reviewed the subject of additive or synergistic effects of two compounds on various microörganisms.

Pulaski and Amspacher[21,22] were the first to show that a combination of streptomycin and sulfadiazine would provide specific therapy for human brucellosis.

After Jones, Metzger, Schatz, and Waksman[13] employed chicken embryos to assess streptomycin activity on Brucella, Hall and Spink[12] and Shaffer and Spink[25] reported on a modification of this technique for testing anti-Brucella compounds. These authors have confirmed the results of Pulaski and Amspacher and have shown that sulfadiazine potentiates the activity of streptomycin, the combination eliminating Brucella from the host. Applying these findings to human patients, Spink *et al.*[26] have shown that a high proportion of acute cases of brucellosis can be cured. Eisele and

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TABLE I—Efficacy of Streptomycin, Sulfadiazine, NaPAB, and Combinations of Streptomycin-Sulfadiazine and Streptomycin-NaPAB in the Treatment of Brucellosis in Guinea Pigs

		Lesions		Titers			
		Severity of	Per cent with	Average Initial	Average Final	Per cent with Initial	Per cent with Final
1)	Controls	Marked 4+	100	1 : 400	1 : 400	100	100
2)	Treatment initiated one week prior to infection Treated 5 times/day NaPAB	Marked 4+	100	1 : 400	1 : 400	100	100
3)	Treatment at time of infection						
	a) 5 *times/day*						
	1—Streptomycin	3+	100	1 : 100	1 : 180	50	80
	2—Sulfadiazine	2+	80	1 : 50	1 : 80	20	60
	3—NaPAB	3	100	1 : 300	1 : 400	100	100
	4—Streptomycin-NaPAB	2	90	1 : 100	1 : 125	80	80
	5—Streptomycin-Sulfadiazine	0	0	0	0	0	0
	b) 2 *times/day*						
	1—Streptomycin	3	100	1 : 200	1 : 200	60	80
	2—Sulfadiazine	2	90	1 : 100	1 : 100	60	70
	3—Streptomycin-Sulfadiazine	0	10	1 : 50	1 : 25	40	0
	c) 1 *time/day*						
	1—Streptomycin	3	90	1 : 100	1 : 125	90	90
	2—Sulfadiazine	1	80	1 : 100	1 : 25	70	50
	3—Streptomycin-Sulfadiazine	0	0	1 : 25	0	60	0
4)	Treatment started two days after infection						
	a) 5 *times/day*						
	1—Streptomycin	2	90	1 : 150	1 : 100	100	90
	2—Sulfadiazine	2	70	1 : 100	1 : 50	80	70
	3—Streptomycin-Sulfadiazine	2	30	1 : 25	1 : 25	20	30
	b) 2 *times/day*						
	1—Streptomycin	2	90	1 : 200	1 : 200	70	90
	2—Sulfadiazine	2	80	1 : 100	1 : 100	90	90
	3—Streptomycin-Sulfadiazine	1	20	1 : 100	1 : 50	40	30
	c) 1 *time/day*						
	1—Streptomycin	3	100	1 : 100	1 : 100	90	80
	2—Sulfadiazine	2	70	1 : 75	1 : 75	70	70
	3—Streptomycin-Sulfadiazine	1	20	1 : 50	1 : 50	50	10
5)	Treatment initiated five days after infection						
	a) 5 *times/day*						
	1—Streptomycin	2	80	1 : 75	1 : 75	100	80
	2—Sulfadiazine	1	70	1 : 100	1 : 50	90	70
	3—Streptomycin-Sulfadiazine	1	50	1 : 125	1 : 25	80	40
	b) 2 *times/day*						
	1—Streptomycin	2	80	1 : 100	1 : 100	90	80
	2—Sulfadiazine	1	60	1 : 100	1 : 50	100	60
	3—Streptomycin-Sulfadiazine	1	20	1 : 100	1 : 50	60	20
	c) 1 *time/day*						
	1—Streptomycin	2	100	1 : 75	1 : 150	100	80
	2—Sulfadiazine	1	40	1 : 100	1 : 25	80	40
	3—Streptomycin-Sulfadiazine	1	20	1 : 75	1 : 25	80	20
6)	Treatment initiated ten days after infection						
	a) 5 *times/day*						
	1—Streptomycin	2	90	1 : 200	1 : 200	100	100
	2—Sulfadiazine	1	70	1 : 200	1 : 100	90	90
	3—Streptomycin-Sulfadiazine	1	20	1 : 150	1 : 50	90	20
	b) 2 *times/day*						
	1—Streptomycin	2	80	1 : 200	1 : 150	100	80
	2—Sulfadiazine	2	80	1 : 150	1 : 150	100	70
	3—Streptomycin-Sulfadiazine	1	10	1 : 100	1 : 100	90	10
	c) 1 *time/day*						
	1—Streptomycin	3	70	1 : 100	1 : 150	100	80
	2—Sulfadiazine	1	70	1 : 100	1 : 50	90	70
	3—Streptomycin-Sulfadiazine	1	20	1 : 100	1 : 25	100	20

McCullough[8] also reported the efficacy of this combination in brucellosis in man.

The investigations reported herein relate to a comparison of the effect of streptomycin alone, sulfadiazine alone, and a combination of these two compounds on brucellosis in male guinea pigs. Trials were also made on the therapeutic value of NaPAB alone and in combination with streptomycin. Studies were made on the optimum number of injections per day and the efficacy of treatments initiated at the time of infection and at various intervals after infection. The entire study was conceived and executed independently of the aforementioned results.

METHODS

Male guinea pigs, of 400 to 500 Gm. body weight, were used throughout this series of trials. Each animal was inoculated intraperitoneally with approximately 1 million cells of a 24-hour culture of *Br. abortus* strain 2308.

The animals were grouped into lots of 10 and were treated according to the following schedule:

1) Controls—infected; no treatment.
2) Treatment initiated one week prior to infection.
   a) 200 mg. NaPAB 5 times/day.
3) Treatment initiated at the time of infection.
   a) Five equal treatments per day at 8 a.m., 11 a.m., 2 p.m., 5 p.m., and 8 p.m.
      Group 1—5,000 units of streptomycin per day.
      Group 2—120 mg. sodium sulfadiazine per day.
      Group 3—1 Gm. NaPAB per day.
      Group 4—5,000 units of streptomycin plus 120 mg. of sodium sulfadiazine per day.
      Group 5—5,000 units of streptomycin plus 1 Gm. of NaPAB per day.
   b) Two equal treatments per day at 8 a.m. and 6 p.m.
      Group 1—5,000 units of streptomycin per day.
      Group 2—120 mg. of sodium sulfadiazine per day.
      Group 3—5,000 units of streptomycin plus 120 mg. of sodium sulfadiazine per day.
   c) One treatment per day at 8 a.m.
      Group 1—10,000 units of streptomycin.
      Group 2—120 mg. of sodium sulfadiazine.
      Group 3—10,000 units of streptomycin plus 120 mg. of sodium sulfadiazine.
4) Treatment initiated two days after infection.
   a) Same groups as in 3, except that no groups were treated with NaPAB or with NaPAB plus streptomycin.
5) Treatment initiated five days after infection.
   a) Same as 4.
6) Treatment initiated ten days after infection.
   a) Same as 4.

In each instance, the $CaCl_2$ complex of streptomycin was administered subcutaneously in 0.5-cc. doses. Both sulfadiazine and para-aminobenzoic acid (PABA) were converted to the sodium salts and given by mouth; each was pipetted in 0.5-cc. doses.

In all cases, the treatment was continued for twenty-one days. On the twenty-second day, the animals were bled for the determination of Brucella agglutination titers. Twenty days later, the animals were sacrificed and blood was collected. Gross pathology was noted; comparable samples of the liver, spleen, lymph nodes, and epididymis were macerated and smeared on tryptose agar plates. The plates were checked after six days for the presence of Brucella colonies, and the relative numbers of the colonies were noted.

Although each group of animals in this series of trials had but 10 animals in any one experiment, repeated trials were made in subsequent experiments. Hence, each group represented in the treatment schedule consisted of 20 animals. Three groups of infected controls were run with 10 animals in each group. The total number of animals in these trials was 810.

RESULTS

The results of the experiments are shown in table 1.

Lesions, as defined here, represent those gross changes observed at autopsy and confirmation of these by the density of Brucella colonies on the agar plates. Except in cases of questionable lesions, there was very good correspondence between the gross signs and the density of recovered organisms.

The figures for the average agglutination titers represent the average for all animals on a phase of study, even though some may have had no titers. Initial titers refer to those determined at the time of cessation of treatment. Final titers refer to those determined twenty days later, at autopsy.

All controls were severely infected, as judged by the size of the spleen and lymph nodes, and by the liver lesions and the many infections of the epididymis and testis. Titers were uniformly high and bacterial isolations were plentiful.

Using these criteria, NaPAB, given for one week prior to the inoculation of Brucella, or at the time of inoculation, proved to give no significant protection. The combination of NaPAB and streptomycin is better than NaPAB alone, but no better than streptomycin alone.

Streptomycin, injected alone, even at the time of infection proved to be of little value in arresting the course of the infection. This is in contrast to the results obtained by Live, Sperling, and Stubbs.[20] These authors report using 1,000 infective cells. Preliminary trials on this problem in our laboratory demonstrated that, under the conditions of our experiment, approximately 1,000 infective cells produced lesions and recoverable cells in 60 per cent of our non-treated controls. In no cases were lesions marked nor was there a high colony count of Brucella. Under these conditions, streptomycin alone, at a level of 5 mg. per day, proved to be highly efficacious. The differences, then, may, in some measure, be attributed to the size of the inoculum.

In view of the promising early results on streptomycin, it was surprising to see that sulfadiazine, at the concentration used here, gave slightly but consistently superior results. Histologic examinations of the kidneys of sulfadiazine-treated animals indicated that the dosage schedule did not cause organ damage.

In all trials, the combination of streptomycin and sulfadiazine proved to be better than either drug alone. The efficacy of the combination was most pronounced when treatment was started at the time the animals received the infecting organisms. In groups treated two and five days after infection, only 4a and 5a showed less than 80 to 90 per cent tissue sterilization.

Treatment initiated ten days postinfection produced results as good as those initiated two days after infection, and one treatment each day proved to give as good results as two or five treatments per day.

DISCUSSION

In view of the bacteriologic cures obtained in human patients with a combination of streptomycin and sulfadiazine, the results obtained in this series of trials are not wholly unexpected. It is dangerous, of course, to extrapolate results from 1 animal to another or to extrapolate from therapeutic results obtained in acute brucellosis to the chronic disease. Simulation of the chronic and acute phases of brucellosis in guinea pigs is difficult with *Br. abortus* because of the fairly high percentage of spontaneous recoveries in this animal. In the experiment cited above, the animals showed definite roughening of the coat and inactivity on the seventh or eighth day postinfection. Those animals in which treatment was initiated ten days after infection could be classified as heavily infected prior to therapy. In the groups treated on the day of infection, the combination of drugs plus a mobilization of the body defenses probably prevented the initiation of the disease.

The literature is replete with references to the efficacy of high blood levels of penicillin or streptomycin. The maintenance of a constant level of drug in the body requires special injection vehicles or, as is the case with penicillin, the concomitant ingestion of a drug, such as caronamide, to prevent loss via the urinary route. From a practical veterinary viewpoint, constant drug levels would be difficult to maintain. It is significant, therefore, that under the conditions of this series of trials, one injection per animal per day yielded as good results as an around-the-clock injection schedule.

SUMMARY

Streptomycin and sulfadiazine given subcutaneously and orally, respectively, have proved to be effective in producing bacteriologic cures in experimental brucellosis in guinea pigs. The combination was as effective when given 10 days after inoculation as when given on the day of infection. It was as effective when given once a day as five times a day.

No toxicity was observed in treated animals; random sampling of the kidneys at autopsy did not yield evidence of sulfonamide damage.

References

[1]Berman, D. T., Irwin, M. R., and Beach, B. A.: The Effect of Penicillin on Cows Infected with *Brucella Abortus*. Cornell Vet., *36*, (1946): 311-313.

[2]Bunnell, D. E., Hutchings, L. M., and Donham, C. R.: Effects of Penicillin on *Brucella Suis in Vitro* and *in Vivo*. Am. J. Vet. Res., *8*, (1947): 367-373.

[3]Chinn, B. D.: The Use of Sulfanilamide in Experimental Brucellosis. J. Infect. Dis., *64*, (1939): 78-82.

[4]Coles, E. H., Jr.: The Sensitivity of Bacteria of Animal Origin to Streptomycin *in Vitro*. Am. J. Vet. Res., *9*, (1948): 152-157.

[5]Cotton, C. M., and Swope, R. E.: Brucella Chemotherapy. I. Studies of the Effect of Para-Aminobenzoic Acid on Brucella *in Vitro*. Am. J. Vet. Res., *9*, (1948): 164-168.

[6]Cotton, C. M., and Swope, R. E.: Brucella Chemotherapy. II. Para-Aminobenzoic Acid in the Treatment of Experimental Brucellosis in Guinea Pigs. Am. J. Vet. Res., *9*, (1948): 169-172.

[7]Dumaresq, J. A.: Effect of Sulphanilamide on *Brucella Abortus* Infection in Guinea Pigs and Cows. Australian Vet. J., *16*, (1940): 102-107.

[8]Eisele, C. W., and McCullough, N. B.: Combined Streptomycin and Sulfadiazine Treatment in Brucellosis. J. A. M. A., *135*, (1947): 1053-1055.

[9]Finch, G. H.: Streptomycin Therapy in Undulant Fever. Am. J. Med., *2*, (1947): 485.

[10]Gilman, H. L., and LeGrow, W. R.: Streptomycin in the Treatment of Experimental Brucellosis in Guinea Pigs. Am. J. Vet. Res., *8*, (1947): 192-195.

[11]Hall, W. H., and Spink, W. W.: *In Vitro* Sensitivity of Brucella to Streptomycin: Development of Resistance During Streptomycin Treatment. Proc. Soc. Exptl. Biol. and Med., *64*, (1947): 403-406.

[12]Hall, W. H., and Spink, W. W.: Therapy of Experimental Brucella Infection in the Developing Chick Embryo. I. Infection and Therapy via the Allantoic Sac. J. Immunol., *59*, (1948): 379-391.

[13]Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A.: Control of Gram-Negative

Bacteria in Experimental Animals by Streptomycin. Science, *100*, (1944): 103-104.

[14]Kelly, E. H., and Henley, T. H., Jr.: Streptomycin in Experimental Brucellosis. J. Bact., *54*, (1947): 80.

[15]King, E. S., and Lucas, M.: Sulfapyridine in Experimental Brucellosis. J. Lab. and Clin. Med., *26*, (1948): 616-621.

[16]Kolmer, J. A.: The Synergistic or Additive Activity of Chemotherapeutic Compounds. Am. J. M. Sc., *215*, (1948): 136-148

[17]Kolmer, J. A., and Rule, A. M.: The Therapeutic Activity of Sulfanilamide and Allied Compounds in Experimental Brucellosis in Mice. J. Pharmacol. and Exptl. Therap., *68*, (1940): 406-412.

[18]Live, I., Stubbs, E. L., and Gardiner, M. R.: The Effect of Sulfapyridine on Cows Known to Eliminate *Brucella Abortus* in the Milk. Am. J. Vet. Res., *4*, (1943): 276-286.

[19]Live, I., Stubbs, E. L., and Gardiner, M. R.: The Use of Sulfathiazole in Cows Known to Eliminate *Brucella Abortus* in the Milk. North. Am. Vet., *24*, (1943): 661.

[20]Live, I., Sperling, F. G., and Stubbs, E. L.: Effect of Streptomycin on Experimental Brucellosis in Guinea Pigs. Am. J. M. Sc., *21*, (1945): 267-272.

[21]Pulaski, E. J., and Amspacher, W. H.: Streptomycin Therapy in Brucellosis. Bull. U. S. Army Med. Dept., *7*, (1947): 221-225.

[22]Pulaski, E. J., and Amspacher, W. H.: Streptomycin Therapy for Certain Infections of Intestinal Origin. New England J. Med., *237*, (1947): 419-428.

[23]Santivanez, J.: The Antibacterial Action of Streptomycin. Cornell Vet., *36*, (1946): 214-221.

[24]Schuhardt, V. T., Rich, G. C., and Beal, G. A.: Sulfadiazine Therapy in Bovine Brucellosis. J. Bact., *50*, (1945): 127.

[25]Shaffer, J. M., and Spink, W. W.: Therapy of Experimental Infection in the Developing Chick Embryo. II. Infection and Therapy via the Yolk Sac. J. Immunol., *59*, (1948): 393-403.

[26]Spink, W. W., Hall, W., Shaffer, J. M., and Braude, A. I.: Human Brucellosis—Its Specific Treatment with a Combination of Streptomycin and Sulfadiazine. J. A. M. A., *136*, (1948): 382-387.

[27]T'ung, T.: *In Vitro* Action of Penicillin Alone and in Combination with Sulfathiazole on Brucella Organisms. Proc. Soc. Exptl. Biol. and Med., *56*, (1944): 8-11.

[28]Wilson, G. S., and Maier, I.: Treatment with Sulfapyridine (M & B 693) of Guinea Pigs Infected with *Brucella Abortus*. Brit. M. J., *1*, (1940): 47-50.

Irradiation of Foot-and-Mouth Disease Virus with Ultraviolet Rays

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RECENT REPORTS indicate that ultraviolet (UV) irradiation transforms some viruses into nontoxic modifications (vaccines) which, nevertheless, retain immunogenic properties. By this method, vaccines have been prepared from rabies and psittacosis viruses. In mice experiments, the rabies vaccine seems to be more active than the desiccated or the chemically inactivated (phenolized) vaccine now in common use.

Our experiments were performed with foot-and-mouth disease (FM) virus, UV inactivated, to study the immunizing properties of the vaccine and for comparison with the standard Schmidt-Waldmann vaccine. Because there is no true method for standardizing FM vaccine, a comparison of even two vaccines (while remaining rather circumstantial) is time consuming and very exacting when guinea pigs and cattle are used.

MATERIALS AND METHODS

A silica tube 5 mm. in diameter and 90 cm. long is placed in the center of a triangular stainless steel stand. At each corner, three Westinghouse sterilamps (WL, 80 cm. long) are placed in such a position that they are equidistant from one another (120 degrees) and 15 mm. from the silica tube. To protect the workers, the entire apparatus is enclosed in a removable steel jacket. At the top of the silica tube is a separating funnel with an electrically driven agitator. At the bottom, the tube is drawn to a fine nozzle, fitted with rubber tubing. Flow from the tube is controlled with a stopcock.

Irradiation time is measured by filling the funnel with water, opening the cocks only long enough to fill the silica tube, and lighting the lamps. The stopcock is opened wide and the time required to pass 100 cc. of water through the tube is measured with a stop watch. Irradiation time can be varied by adjusting the flow with the stopcock.

Guinea-pig or cattle lymph, containing FM virus (1.4%) adsorbed on aluminum hydroxide, was mixed with an equal volume of aluminum hydroxide solution (1.8% Al_2O_3). This is the same basic material as employed in making Schmidt-Waldmann (S.-W.) vaccine. The S.-W. vaccine is then formolized, heated at 26 C. for sixty hours, and homogenized for use.

In this experiment, the FM lymph was diluted with 9 parts of freshly distilled, CO_2-free water. The agitator was set in motion, the bulbs were lighted, and 100 cc. of the liquid was allowed to flow into the collecting beaker. The period of irradiation was varied by adjusting the rate of flow.

EXPERIMENTAL

Experiments with Guinea-Pig Virus.—The virus used was of type A Vallée, and the titer was 10^{-7}, which was the dilution providing the smallest quantity of virus capable of inducing FM when injected intracutaneously into guinea pigs. Preliminary tests had shown that infectivity was lost at a specific time of irradiation, but that power to immunize was retained beyond this time and then lost gradually as irradiation time became longer. In other words, it had become apparent that irradiation time could be so short as to fail to inactivate all of the virus, or it could be so long as to destroy not only infectivity but also all immunogenic properties. But, by properly timing the UV irradiation, a nontoxic adsorbate of highly immunizing power was produced —the FM virus was transformed to vaccine. In one typically severe experiment, of the many performed, 6 guinea pigs were each injected with 1 cc. of this irradiated vaccine intramuscularly and, in addition, each received 3 intracutaneous plantar injections. Results showed that infectivity had been lost at irradiation exposures of more than sixty-five seconds, while immunogenic powers were retained at exposures up to 540 seconds, the longest exposure time studied.

Experiments with Cattle Virus.—Again, only a single typical experiment, involving but a few animals, will be described. Cattle FM virus must be used, since guinea-pig virus does not induce a uniform response. In fact, results are somewhat irregular even with cattle virus.*

Pooled lymph was used. This was collected twenty-four hours after injection from intra-

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*This is rather similar to what we know of rabies. Rabbit-passage virus (virus fixé, Pasteur) and street virus (virus from a spontaneous case of rabies in a dog, cat, cow, etc.), display mutually complicated immunologic peculiarities which make their study difficult.

lingual blisters on 3 cows, and possessed a cattle titer of 10^{-7} and a guinea-pig titer of 10^{-9}. The adsorbate contained 1.4 per cent virus and 50 per cent $Al(OH)_3$; it was diluted in 9 parts water and UV irradiated for varying periods. The pH was 6.5 before irradiation, and was not adjusted later, remaining slightly acid. Injections into guinea pigs were as described previously.

No sharp line could be drawn, but it was apparent that exposures of less than forty-five seconds were ineffective; all guinea pigs exposed developed the disease. At sixty seconds of UV irradiation, slight symptoms of FM developed in 76 test guinea pigs, while, at 165 seconds, toxicity was lost. No symptoms were seen, but immunity was induced in all guinea pigs. However, at 300 and also at 390 seconds of irradiation, half of the injected guinea pigs developed FM symptoms.

To test the value of vaccines on cows, the two samples giving the best results in guinea pigs were tested on cattle. Using the two lots of virus which had been irradiated for sixty and for 165 seconds, 3 cows were vaccinated with each lot. Each cow was injected intralingually with 1 cc. of the vaccine, and also injected intramuscularly with 100, 60, or 30 cc. of vaccine. The cow receiving only 30 cc. of the 165-second vaccine developed moderate, generalized FM. This cow was not tested for immunity, but each of the other 5 was challenged fourteen days later with an intralingual injection of 10,000-infecting doses. Only 1 showed a moderate local reaction, without general symptoms, but 2 susceptible control animals developed general FM symptoms.

The results agree fairly well with the guinea-pig results. Infectivity was not completely lost under the extreme severity of the challenge, but the immunity engendered was stronger than in the guinea pigs. The efficacy of the adsorbate vaccine was later emphasized when only 3 instead of 30 cc. of diluted vaccine protected against a challenge dose. Even the 30 cc. (equal to 3 cc. of undiluted vaccine) was regarded as remarkably low dosage.

Irradiation of Virus Diluted with Saline, Without Aluminum Hydroxide.—Experiment with Virulent Lymph.—A simple dilution of virulent lymph appears to behave in the same manner as diluted adsorbate, but undiluted adsorbate requires longer irradiation, to produce a vaccine, than doses of the diluted fluid. Since this is so, only a few experiments will be described, although many were performed.

When virulent lymph was diluted with saline to 11.2, 5.6, and 2.8 per cent, respectively, it was learned that the heavier suspensions required longer irradiation to inactivate. The lowest virus content (2.8%) failed to produce FM symptoms after 117 seconds of UV irradiation, while the highest virus content (11.2%) was not completely inactivated at the longest exposure (397) seconds) used. Half of the animals inoculated with this product developed FM. It is likely that the determining factor in this connection is the amount of protein in the solution rather than the amount of virus.** The immunity produced was satisfactory; all experimental animals not developing FM also resisted challenge exposure to live virus.

Comparison Between Plain and Adsorbed Irradiated Vaccines.—The immunizing effect of FM virus, inactivated with a combination of formalin and heat, and its enhancement by adsorption to aluminum hydroxide, were reported by Schmidt and Hansen[1] (p. 8). This followed similar work with diphtheria toxin. Moreover, the adsorbed vaccine produces an immunity which is much more lasting than that produced by unadsorbed vaccine. The response of plain and adsorbed virus to irradiation was therefore studied.

In order to secure larger portions of good antigen, 8 lots of 100 cc. each were prepared as follows: Virus having a titer of 1 : 10 million was diluted to 2.8 per cent and irradiated for periods varying from forty-eight to 530 seconds. Although the results are not completely regular, infectivity is destroyed at about 200 seconds, and 197 seconds was chosen as the arbitrary irradiation time for future experiments. One animal developed mild lesions upon challenge, following inoculation with material exposed to UV rays for 530 seconds, thus, raising the question whether this was approaching the level at which immunogenic properties would be completely destroyed. This question remains unanswered.

Challenge doses were commonly injected at the end of one week, and sometimes as early as the fifth day. However, challenge doses were administered at from five to eighty days after vaccination. The immunity produced by the adsorbate vaccine persisted longer than that from the plain vaccine, but complete results are not yet available.

The preceding results have all followed use of virulent lymph. Since the so-called aphtha cover also contains large amounts of virus and is used in the preparation of commercial vaccine, it, too, was extracted, irradiated, and tested. The results were analogous to those reported above.

Virulent serum is sometimes used in vaccine production, but contains much less virus than the lymph (possibly as little as 1%). Despite this fact and certain practical problems, the effect of irradiation on virus-containing serum was studied. In guinea pigs, the virus concentration in the blood is highest at twenty-four to thirty hours following injec-

**When formalin is used as the inactivating agent, the amount of protein present has little, if any, effect on the rate of inactivation.

tion, *i.e.*, simultaneously with the full ripening of the primary aphthae. In cattle, the maximum virus concentration is reached at thirty to thirty-six hours.

Guinea-pig serum having the unusually high titer of 1 : 100,000 was used directly, and diluted 1 : 1 and 1 : 10. The undiluted and the 1 : 1 diluted serum remained infective even after 675 seconds irradiation, but the 1 : 10 dilution was inactivated at 270 seconds of exposure, while retaining its immunogenic properties to 391 seconds of irradiation. In general, the irradiated blood serum was a better antigen than irradiated lymph.† Whether serum protein exerts an effect similar to aluminum hydroxide remains a question. Early British workers[1] (p. 12) added serum, casein, and bacterial vaccines to formolized vaccines, but the results were inconclusive. Nevertheless, the utilization of blood serum as vaccine deserves study, since the amount of vaccine potentially available from this source would be considerable.

Adjusting the pH.—Although Waldman and Köbe worked with a pH as high as 10 and suggested stabilizing the vaccine at pH 9 with glycocoll-sodium, later work by British investigators (and verified in Denmark) shows the optimum to be about pH 7.6, with a range from 7 to 8. The otherwise excellent phosphate buffer tends to raise the pH to more than 8, at which point the virus may go through a process of partial elution. Other useful buffers are difficult to find, but we have experimented extensively with the phosphate content of the buffer. When phosphate buffer at pH 7.8 is added to aluminum hydroxide, we have found it necessary to deviate from Sörensen's standard mixture (m/15) in order to hold the pH constant. In our experiments, a variation to m/500 has enabled us to hold the pH reasonably constant, and this has been sufficient phosphate to retain the immunizing power of the vaccine.

The precise reaction when primary and secondary phosphate ions become alkaline is not clear. At one time, we believed that the primary ions were adsorbed to a greater extent, but if so, the pH should not remain constant at the low ion concentration. Use of buffers with a lower pH (7.2) has not prevented the mixture from becoming alkaline when the salt concentration remains at a level of m/50. Aluminum hydroxide has no buffer effect, and, therefore, virus adsorbates without buffer will fall to an acid pH of 6.5, or even lower. However, this drawback should be avoided by supplying a buffer with a phosphate concentration of about m/500.

†The objection might be raised that it is not the virus, modified by irradiation, that has caused the immunity, but possible small remnants of toxin. However, the same objection might be raised, and with equal right, in respect of all the experiments, inasmuch as 6 animals on the whole is too small a number. If one used 100 or 500 animals, a particularly sensitive individual might react. The same would also apply to the vaccine now used. But, during the fifteen years of experimental FM research at this institute, we have had several hundreds of examples that animals not reacting to an intracutaneous injection of active virus are very rarely immune. But, if they are, it is a natural and not a specific immunity, for the blood contains no antibodies. Even animals with an atypical (abortive) take at the injection site, or indeed, a small primary aphtha, are not definitely immune. In the latter case, however, it is the rule. The position is partly analogous to what is observed in the case of toxins and toxoids (anatoxins). The antigen is identical with the nontoxic modification.

SUMMARY

1) When FM virus was irradiated with ultraviolet rays (ultra-short waves) under suitable experimental conditions, it passed into a noninfective modification (anavirus) possessing pronounced immunizing properties.

2) When adsorbates were prepared from virus samples so treated, we obtained vaccines with a more powerful antigenic effect, which produced an immunity of longer duration than anavirus alone.

3) Adsorbates prepared from active virus and aluminum hydroxide were transformed, in the same manner, to vaccines of high quality.

4) Virus for the preparation of vaccine by irradiation could be used either in the form of diluted lymph or diluted serum. The latter had a relatively higher immunizing effect than the former, having regard to the virus content in the two initial materials.

5) The effectiveness of the irradiation depended upon the degree of dilution of the material. Samples of concentrated virus were more difficult to inactivate than diluted. The same applied to virus adsorbates. The concentration of aluminum hydroxide also played a role in inactivation.

References

[1]Galloway, I. A., and Nicolau, S.: Third Rep. Foot-and-Mouth Dis. Comm., London, Eng., (1928):128.

[2]Galloway, I. A.: Fourth Rep. Foot-and-Mouth Dis. Comm., London, Eng., (1931):331.

[3]Habel, R., and Sockrider, B. T.: J. Immunol., *56*, (1947):273.

[4]Habel, R.: Public Health Reports, *62*, (1947):791.

[5]Francis, R. D., Milzer, A., and Gordon, F. B.: Proc. Soc. Exptl. Biol. and Med., *66*, (1947):184.

Studies on Johnin

VII. Frequency of Intradermal Testing as Related to Local Desensitization

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A STUDY of various animals was made by Johnson and Cox[1] and Johnson[2] to determine which species of animal was most suitable for testing johnin allergins. To be suitable for this purpose, an animal should remain sensitive to the johnin allergins for several months, after having been sensitized by the intraperitoneal injection of a mineral oil suspension of *Mycobacterium paratuberculosis*. They concluded, as a result of studies on rabbits, guinea pigs, chickens, and cattle, that cattle were best suited for this type of work.

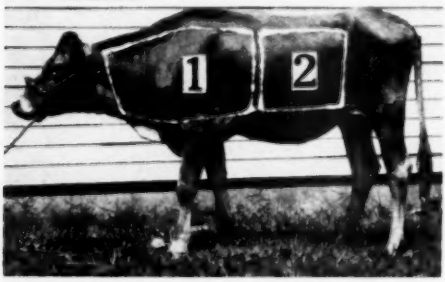
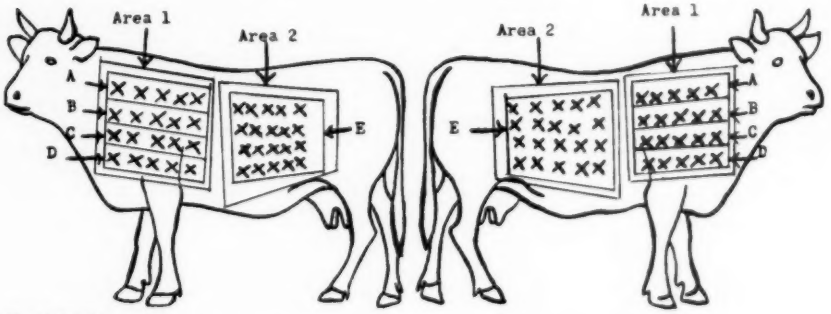


Fig. 1—One of 3 Jersey steers showing areas selected for injection sites.

Since the conclusion of those studies, sensitized cattle have been used in the experimental work on Johne's disease at the regional laboratory, particularly for testing the specificities and potencies of experimental johnins. Once sensitized, such cattle are used in three or more successive tests. Since it has been generally believed that the first injection of johnin may produce a local desensitization of indefinite duration, and thus interfere with reactions produced by subsequent injections in the same site, it seemed desirable to ascertain the shortest interval between tests or injections that would insure disappearance of local desensitization and thus eliminate interference.

The time that should elapse between successive tests is important not only in experimental work on artificially sensitized cattle, but also in the practical application



X = injection sites.
Region A = sites injected at weekly intervals.
Region B = sites injected at 2-week intervals.
Region C = sites injected at 3-week intervals.
Region D = sites injected at 4-week intervals.
Region E = control area, only one injection made on each site.

Fig. 2—Regions showing sites of injections at weekly intervals, and at two-, three-, and four-week intervals. Region E is the control area.

From the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Auburn, Ala.

of the johnin and tuberculin tests under field conditions. Ninety days is generally accepted as the shortest interval at which

intradermal tests may be repeated. With that interval, several years have been required, in some herds, to reach a point where a negative test was given by all the animals in the herd. If this interval between tests could be successfully shortened, it would seem that the time required to detect and eliminate infected animals could also be shortened and thus permit greater progress to be made in the eradication of the disease. In order to determine the time required to effect recovery from local desensitization, the work described below was carried out.

PROCEDURE

Three grade Jersey steers, 1½ years of age, weighing about 500 lb. each, and artificially sensitized according to Johnson,[2] were used as test animals. One additional steer of the same age and size, but not sensitized, was included as a control. Two areas (fig. 1 and 2) were outlined on each side of each animal. Forty injection sites, 20 on each side, were selected in area 1. These sites were spaced 4 in. apart in order to prevent a large reaction from extending to an adjacent injection site. They were clipped and occupied the positions indicated by the x's of regions A, B, C, and D within area 1 (fig. 2). Each site was injected intradermally one or more times, as described below, with 0.2 cc. of johnin. Earlier work of a similar nature had shown that a large number of injections on 1 animal had no effect on the general health of the test animals. In order to determine whether or not johnin produced a reaction, the thickness of the skin at each site was measured before injection and forty-eight hours after injection. Measurements were made with a dermal thickness gauge.[3] Increases in thickness of less than 1 mm. were classed as negative; those 1 mm. to 2.75 mm. as suspicious; and those 3 mm. or more as positive. The intervals between injections and the number of injections at each site were as follows: Ten sites in region A (fig. 2), on each of the 3 animals, were injected once each week until 10 tests had been completed on each site, making a total of 100 tests per animal in this region. At the end of ten weeks, the formation of fibrous tissue in the region that had been injected at weekly intervals had developed to the stage that further injections were not attempted. Ten sites on each animal in region B (fig. 2) were injected every two weeks until 10 tests per site had been completed, or a total of 100 tests per animal in this region. Ten sites on each animal in region C (fig. 2) were tested every three weeks until 6 tests on each site had been completed, making a total of 60 tests per animal in region C. Ten sites on each animal in region D (fig. 2) were tested every four weeks until a total of 5 tests on each site had been completed, or a total of 50 tests per animal in the last region.

Area 2 (fig. 1 and 2) was used for control injections. Forty injection sites were selected on this area, 20 on each side. These sites were clipped as they were used, and occupied positions designated by the x's in region E. One injection of 0.2 cc. of johnin was made into one of these sites on both sides of every animal at weekly intervals. New sites were used each week for twenty weeks, so a total of 40 tests were made in this area on each animal. All reactions in area 2 were recorded by the same method used for those in area 1.

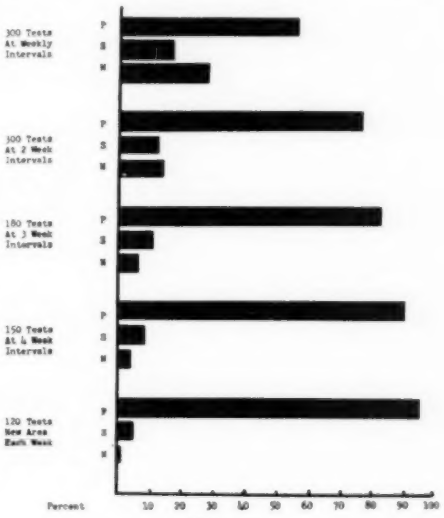


Figure 3

P = positive; S = suspicious; N = negative.

RESULTS

The results shown in per cent of positive, suspicious, and negative reactions are tabulated in figure 3. These results are based on the following total number of readings: One-week intervals, 300; two-week intervals, 300; three-week intervals, 180; four-week intervals, 150. The negative control animal gave no reaction at any time during the experiment, although it received the same number of injections as each of the other animals.

When a new site was used each week, about 97 per cent of the reactions were classed as positive and about 3 per cent were suspicious. However, when a site injected one week was reinjected at weekly intervals, the percentage of positive reactions was considerably less—about 55 per

cent—with 15 per cent of suspicious reactions and about 30 per cent of negative reactions. As the interval between injections was increased, the percentage of positive reactions on injected sites increased. Thus, when the interval was two weeks, the positive reactions were about 75 per cent; when four weeks, about 87 per cent.

CONCLUSIONS

When the interval between injections on artificially sensitized cattle was four weeks, desensitization, which had been exceedingly high at one-week intervals, had been almost, but not entirely, eliminated. However, an injection made at one site had no influence on the results of injections at new sites made at the same time or later.

References

[1]Johnson, H. W., and Cox, B. F.: Studies on Johnin. Am. J. Vet. Res., *3*, (1942):131-138.

[2]Johnson, H. W.: Studies on Johnin. III. Multiple Testing on Sensitized Cattle as a Method of Studying Johnin Potency. Am. J. Vet. Res., *5*, (1944):179-188.

[3]Kummer, F. A., and Johnson, H. W.: Studies on Johnin. IV. Dermal Thickness Gauge. Am. J. Vet. Res., *5*, (1944):189-192.

Effect of Adjuvants upon the Immunizing Quality of Ether-Killed *Brucella Abortus*

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IT WAS previously reported[1] that injections of *Brucella abortus*, killed with ethyl ether, protected guinea pigs against artificial exposure to virulent *Br. abortus* four to six weeks after immunization. The present study deals with the duration of immunity produced in guinea pigs by the administration of ether-killed Brucella, with and without adjuvants.

Harrison *et al.*[2] and Bell[3] have reported that an alum-precipitated pertussis vaccine prolongs protection against whooping cough. Freund and Bonanto[4] have shown that a suspension of killed typhoid bacilli, combined with falba* and mineral oil,** markedly increases and prolongs the antibody production of this antigen in rabbits. In this study, the influence of the above two adjuvants upon the immunizing effect of killed Brucella cells was investigated.

MATERIALS AND METHODS

Preparation of Ether-Killed Brucella Vaccine.—Recently isolated CO_2-sensitive strains of *Br. abortus* were used in the preparation of the different lots of whole cell vaccine killed with ethyl ether. The cultures were grown on tryptose agar slants under 10 per cent CO_2 tension for forty-eight hours, and the growth was washed off with 0.85 per cent NaCl. The physiologic saline suspension of cells was removed, and centrifuged at high speed for one hour; then the supernatant fluid was poured off. Finally, the sediment was resuspended in 0.85 per cent NaCl, and ethyl ether was added, to the point of saturation. Prior to the addition of ether, a small quantity of the material was removed and diluted, and colony plate counts were made to determine the number of Brucella cells in the original suspension. Twenty-four hours was sufficient to kill the organisms, as was determined by plating the material on tryptose agar.

Preparation of Alum-Precipitated Vaccine.—The alum-precipitated vaccine was prepared, essentially, according to the technique of Harrison *et al.*[2] The suspension of ether-killed *Br. abortus* was diluted to the desired concentration with 0.85 per cent NaCl. Then, to each cubic centimeter of saline suspension were added 0.027 cc. of 10 per cent sodium bicarbonate solution and 0.25 cc. of 4 per cent potassium aluminum sulfate solution, both having been previously filtered through a Berkfeld N candle. Finally, the material was placed in the refrigerator overnight. A flocculent precipitate formed, which was thoroughly resuspended just before use.

Preparation of Saline-in-Oil Emulsion Vaccine.—The oil-emulsion vaccine was prepared according to the technique of Freund and McDermott.[5] The falba and the mineral oil were sterilized in the autoclave. Then, 1 part of the desired dilution of a saline suspension of ether-killed Brucella was emulsified in 1 part of melted falba, to which 3 parts of mineral oil were added, with constant agitation by means of an electric stirrer.

Guinea pigs, weighing about 400 Gm., were employed, and all animals were immunized by the subcutaneous route. The quantity of vaccine and the number of injections in the different experiments varied. At certain intervals after immunization, groups of guinea pigs were bled, and the agglutination titers of the animals which had received a saline suspension of *Br. abortus* were compared with the titers of the animals immunized with an identical suspension of *Br. abortus* combined with an adjuvant. Finally, at given intervals after immunization, groups of guinea pigs, together with controls, were challenged with a freshly isolated CO_2-sensitive strain of *Br. abortus*. The various challenge doses, which were injected subcutaneously, contained from 200 to 500 live *Br. abortus* organisms, according to plate counts on the suspensions at the time of injection. The number of organisms used, as the experimental data will show, was sufficient to produce 100 per cent infection in all but one of the control groups of animals. On several occasions, when higher dilutions of the culture suspensions used in the challenge doses were prepared and injected into small groups of normal guinea pigs, it was found that 10 to 20 organisms were sufficient to infect 50 per cent of the exposed animals.

About six weeks after the animals had been injected with the challenge dose, they were

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*Falba is the trade name of a lanolin-like substance prepared by Pfaltz and Bauer, Inc., New York, N. Y.

**The mineral oil used was Atreol No. 9, a light mineral oil, U.S.P., produced by the Atlantic Refining Co.

sacrificed; and the cut surface of pieces of liver and spleen was rubbed over the surface of tryptose agar plates to determine the presence of Brucella. The plates were incubated for five days; and if no growth of Brucella were obtained from either of the two tissues, the animal was considered negative. In order to determine whether cultures negative after five days of incubation would show Brucella colonies upon further incubation, the culture plates from 16 guinea pigs, with negative findings in one experiment, were reincubated under 10 per cent CO_2 for ten more days. In no instance did the plates show Brucella colonies at the end of the second incubation period.

EXPERIMENTAL

Experiment 1.—One group of guinea pigs received 3 injections of killed *Br. abortus* in saline at three-day intervals. Each injection consisted of 1 cc. of the suspension, which contained 40 billion Brucella cells, according to preliminary plate counts; so that a total of 120 billion killed organisms was administered to each animal. Another group of guinea pigs was given the same doses of the identical vaccine, which had been precipitated by potassium alum. A small number of animals from each group was challenged two, four, six, and seven and a half months, respectively, after the administration of the last immunizing dose. As shown in table 1, there was not a significant difference in resistance between the guinea pigs which had been injected with the alum-precipitated vaccine and those which had been given the vaccine alone, when the animals were challenged two months after immunization. However, the remaining guinea pigs, which were challenged four, six, or seven and a half months after immunization, showed a marked variation in immunity between the two differently treated groups of animals. All control animals yielded *Br. abortus* on cultures. Agglutination tests were carried out on the various groups of guinea pigs just before administration of the test dose of virulent *Br. abortus*. It was found that the animals, injected with the saline suspension of killed Brucella and challenged two months after immunization, had an average agglutinin titer of 1 : 10; and that four, six, or seven and a half months after immunization, none of the animals, injected with the saline suspension of Brucella, had detectable agglutinins in 1 : 10 dilution of their serums. In the case of the animals injected with vaccine adsorbed onto alum, on the other hand, the average agglutinin titer of the guinea pigs challenged two months after immunization was 1 : 70; that of the animals challenged four months after immunization was 1 : 140; and the average titers of the two groups challenged six and seven and a half months after immunization, respectively, were 1 : 14, in each instance.

Experiment 2.—In this experiment, one group of guinea pigs was injected with ether-killed Brucella suspended in saline, and another group was injected with the identical vaccine combined with falba and mineral oil. Each animal was given two simultaneous injections of 1 cc., which contained a total of 100 billion Brucella cells. At various intervals, ranging from four to ten months after immunization, small groups of animals, together with untreated controls, were challenged with virulent *Br. abortus*. As seen from the results

TABLE 1—Immunization of Guinea Pigs with Brucella Vaccine on Alum and in Saline

Months between immunization and challenge	*Br. abortus* challenge dose (No.)	Animals injected with vaccine:		Per cent animals found infected at autopsy when vaccine was used with:	
		On alum	In saline	Alum	Saline
2	350	7	8	0.0	12.5*
4	320	6	6	33.3	66.6*
6	200	7	8	28.5	100.0*
7½	250	6	6	0.0	100.0*

*All control guinea pigs (total 28), injected with the same challenge doses as the immunized animals, were positive on cultures at the time of autopsy.

TABLE 2—Immunization of Guinea Pigs with Brucella Vaccine in Saline-in-Mineral Oil Emulsion and in Saline

Months between immunization and challenge	*Br. abortus* challenge dose (No.)	Animals injected with vaccine:		Per cent animals found infected at autopsy when vaccine was used with:	
		In Oil	In saline	Oil	Saline
4	250	11	10	18.2	30.0*
5½	500	11	9	18.2	55.5*
7	400	10	10	0.0	50.0*
10	250	10	9	10.0	55.5*

*All control guinea pigs (total 42), injected with the same challenge doses as the immunized animals, were positive on cultures at the time of autopsy.

presented in table 2, the number of infected animals in the group injected with the oil-emulsion vaccine varied from 0 to 18 per cent; while the group injected with the vaccine in saline showed infection in 20 per cent of the animals challenged four months after immunization, and in 50 to 55 per cent of the guinea pigs challenged thereafter. All the control animals were found infected at autopsy. The agglutinin titers of the animals injected with the oil emulsion of killed Brucella reached their peak (*i. e.*, an average titer of 1 : 1,500) about two months after immunization; then the titers receded gradually, so that after eight more months the average titer was still 1 : 220. On the other hand, the animals injected with a saline suspension of killed Brucella had an average agglutinin titer of about 1 : 35 three weeks after immunization, after which time it decreased rapidly.

Experiment 3.—Here three groups of guinea pigs were injected with ether-killed *Br. abortus* from the same lot of vaccine which was either (A) combined with falba and mineral oil, (B) adsorbed onto alum, or (C) suspended in saline. The animals received only one subcutaneous injection of 1.5 cc., which contained 60 billion Brucella cells. Thus, in this case, the animals were injected with about half the number of ether-killed organisms, and it declined further during the following one and a half months; so that eleven and a half months after immunization, the resistance of the animals in group B was as low as that of the guinea pigs injected with a saline suspension of the vaccine. Thus, a single injection of an oil emulsion of ether-killed Brucella cells produced effective protection in guinea pigs throughout the period of study in this experiment, while the immunity in the animals injected with an alum-precipitated vaccine was of shorter duration. Whether repeated injections of the vaccine would have prolonged the duration of immunity in the latter group is a question which needs further investigation.

Agglutination tests, carried out at the time of challenge of the various groups of animals, showed that six and a half months after immunization the animals in group A had an average titer of 1 : 106; those in group B had an average titer of 1 : 20; and the guinea pigs in group C were negative in 1 : 10 dilution of their serums. Ten months after immunization, the animals in group A had an average titer of 1 : 90, while those in group B and group C were negative in 1 : 10 dilution of their serums. Finally, eleven and a half months after immunization, the guinea pigs in group A had an average titer of 1 : 56;

TABLE 3—Immunization of Guinea Pigs with Brucella Vaccine in Saline-in-Mineral Oil Emulsion, on Alum, and in Saline

Months between immunization and challenge	*Br. abortus* challenge dose (No.)	Animals injected with vaccine:			Per cent animals found infected at autopsy when vaccine was used with:		
		In oil	On alum	In saline	Oil	Alum	Saline
6½	295	9	8	10	0.0	0.0	50.0*
10	325	10	10	9	20.0	50.0	66.6**
11½	460	11	10	11	9.1	70.0	72.7*

*All control guinea pigs (total 21), injected with the same challenge doses as the immunized animals, were positive on cultures at the time of autopsy.
**Two of 10 control guinea pigs were negative on cultures at the time of autopsy.

as compared with the animals in the preceding two experiments.

In this experiment, the first groups of immunized animals, together with untreated controls, were subjected to a challenge dose of virulent *Br. abortus*, six and a half months after immunization. At this time, as shown in table 3, all guinea pigs from groups A and B were negative on cultures, while 50 per cent of the animals from group C proved infected. Some of the remaining animals in the various groups were challenged ten months after immunization, and the rest one and a half months later. According to the data in table 3, the guinea pigs in group A continued to show a marked resistance to infection throughout the eleven and a half months. On the other hand, the immunity of the animals in group B decreased distinctly between six and a half and ten months after immunization; and the animals in the other two groups were negative to the agglutination test in 1 : 10 dilution of their serums.

Local Reaction at the Site of Injection of Vaccine.—In each experiment, the sites of injection of the vaccine, with and without adjuvants, were examined periodically. Administration of the alum-precipitated vaccine resulted in the formation of firm, small nodules, which usually disappeared within several weeks. The majority of animals inoculated with the oil-emulsion vaccine, on the other hand, developed local swellings which ranged in size from that of a small pea to the size of a hazelnut. The swellings sometimes persisted for months and then decreased gradually. When cut into at autopsy of the animals, these swellings were found to be made up of cysts which contained a thick material comparable in consistency to the original in-

oculum. The injection of ether-killed *Br. abortus* without adjuvants did not cause local tissue reactions.

SUMMARY

This study deals with the question of whether ether-killed *Brucella abortus*, adsorbed on alum or combined with falba and mineral oil, represent a better immunizing agent against infection with *Br. abortus* than the same vaccine without an adjuvant. The results obtained in guinea pigs indicate that either of the two adjuvants combined with the Brucella antigen distinctly enhances its ability to prolong the state of resistance, although the saline-in-oil emulsion of the vaccine proved more efficacious, in that respect, than the vaccine precipitated with alum. From the standpoint of the influence of the adjuvants upon the duration of immunity, problems concerning the most suitable immunizing doses and the best mode of administration of the vaccine need further investigation.

The finding of an efficient vaccine consisting of killed Brucella cells would obviate some of the objections raised against the use of live Brucella vaccines in animals. It could also lead to the development of a much needed immunizing agent against brucellosis in man.

References

[1]Live, L., Sperling, F. G., and Stubbs, E. L.: Studies on the Immunization of Guinea Pigs Against Brucella Infection. J. Infect. Dis., 77, (1945):16-24.

[2]Harrison, W. T., Franklin, J. P., and Bell, J. A.: Prophylactic Value of a Single Dose of Precipitated Pertussis Vaccine. Pub. Health Rep., 54, (1938):793-796.

[3]Bell, J. A.: Pertussis Prophylaxis with Two Doses of Alum-Precipitated Vaccine. Pub. Health Rep., 56, (1941):1535-1546.

[4]Freund, J., and Bonanto, M. V.: The Effect of Paraffin Oil, Lanolin-Like Substances and Killed Tubercle Bacilli on Immunization with Diphtheric Toxoid and *Bacterium Typhosum*. J. Immunol., 48, (1944):325-334.

[5]Freund, J., and McDermott, K.: Sensitization to Horse Serum by Means of Adjuvants. Proc. Soc. Exptl. Biol. and Med., 49, (1942):548-553.

The Pathology of Niacin Deficiency in Swine

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SYMPTOMS and lesions of niacin (nicotinic acid) deficiency in experimental animals have been observed and reported by many research workers since the investigations of Goldberger, Wheeler, and Lillie,[5] with pellagra and pellagra-preventive diets. Some of the early work was complicated by multiple deficiencies, such as that of Miller and Rhoades,[10] who, with a diet supplemented only with thiamin and riboflavin, were able to produce diarrhea, motor weakness of the hind limbs, ulceration of the lips and tongue, gastritis, and hyperchromic macrocytic and hypochromic microcytic anemias in pigs. Birch, Chick, and Martin,[1] using a modified Goldberger diet, produced diarrhea, scurfy, scabby skins, particularly behind the ears, and an inflammation of the large intestine, characterized by a fibrinous cheesy covering of the intestinal mucosa. In a laboratory examination of one herd of pigs, McEwen[9] found lesions of necrotic enteritis in the absence of Salmonella organisms. These lesions were confined to the alimentary canal and were characterized by varying thicknesses of diffuse diphtheritic material on the ridges of the intestinal folds. Lesions found involved both the small and large intestine. Mesenteric lymph nodes, in more severe cases, were enlarged and slightly reddened. Chick and coworkers[3] again produced diarrhea and dermatitis, but this time also caused a paresis of hind quarters. They reported recoveries following injections of niacin. No *Salmonella choleraesuis* organisms were found. Davis and Freeman,[4] feeding a number of rations containing large amounts of corn, were able to produce diarrhea, rough coats, thick, scurfy skin, diffuse necrosis of the mucosa of the cecum and colon, retention of cheesy material in the intestinal lumen, enlargements of glands, and nodule formation. Yeast, liver, alfalfa, and niacin were curative. Hughes,[6] in establishing minimum niacin requirements for pigs, reported inability to rise, dry, harsh skin, and vomiting in deficient animals. Worden and Slavin,[13] using a nicotinic acid-deficient ration, reported blood-stained diarrhea, greenish yellow diphtheritic deposits on the mucous membrane of the cecum and colon, petechial hemorrhages in the mucous membrane, hemorrhagic abdominal lymph nodes, serofibrinous pleurisy, hydrothorax, and hydropericardium. *Salmonella choleraesuis* was recovered from the mesenteric lymph nodes. Wintrobe and coworkers,[12] employing niacin-deficient rations supplemented with pyridoxine, calcium pantothenate, and choline, as well as thiamin and riboflavin, were

TABLE I—Composition of Rations*

	Ration A (%)	Ration B (%)
Corn	87.0	80.0
Casein (commercial)	5.5	12.0
Soybean oil meal	5.5	6.0
Complex mineral mixture	2.0	2.0

*The following amounts of vitamins were supplied daily to each animal: thiamin, 10 mg.; riboflavin, 10 mg.; calcium pantothenate, 25 mg.; and pyridoxine, 6 mg.

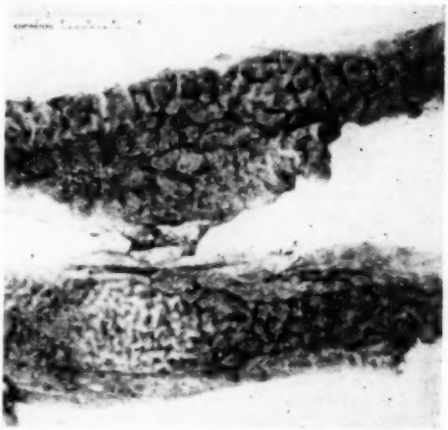

Fig. 1—Portion of the colon demonstrating the adhering fecal masses and thickening of the intestinal wall.

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unable to develop pathologic changes when the protein content of the ration was high. However, when the protein content was low, they were able to demonstrate diarrhea, untidy coats, poor appetites, severe normocytic anemia, some degeneration of posterior ganglion cells, and some chromatolysis of dorsal root ganglia, although no abnormal gaits had been noticed. Braude, Kon, and White,[2] using niacin-deficient rations, reported diarrhea, rough-staring hair, ulceration of the buccal mucosa, and necrotic enteritis of varying extent. No *S. choleraesuis* organisms were isolated. Pigs dying during the experiment showed pneumonic changes. Powick, Ellis, Madsen, and Dale,[11] when feeding rations low in niacin, were able to demonstrate diarrhea and decreased appetite, associated with varying degrees of necrotic enteritis from mild to practically complete occlusion of the whole of the large intestine with a diphtheritic membrane. Some deficient animals were devoid of lesions.

MATERIALS AND METHODS

The pathologic observations discussed in this report were made from materials and data accumulated during the experiments reported by Luecke, McMillen, Thorp, and Tull.[7,8] Niacin-deficient corn, casein, and soybean rations, supplemented with thiamin, riboflavin, calcium pantothenate, and pyridoxine hydrochloride were fed to produce the pathologic changes reported in this paper. The complete rations are shown in table 1.

Tissue sections were taken from the cecum and regions in the colon, corresponding to the ileocecal valve, and 1 ft., 2 ft., 3 ft., and 5 ft. from the ileocecal valve. The tissue blocks were taken immediately at the time of necropsy, and fixed, at once, in Zenker's solution. Sections were embedded in paraffin and stained with hematoxylin and eosin.

RESULTS

Symptoms.—Swine, fed rations containing insufficient quantities of niacin, first showed decreased appetites and decreased daily gains from the third to the fourth

TABLE 2—Average Blood Cell Determinations on Pigs Fed Niacin-Deficient Diets

Group 1	No. of pigs		Normals	28 days	56 days	87 days	Extent of gross lesions
Basal diet B	5	R.B.C.	6,330*	5,818	4,712	6,135	None to extensive
		W.B.C.	16,040	22,580	25,008	18,700	
Basal diet B	5	R.B.C.	6,640	5,638	6,380	7,547	None to mild
B vitamins**		W.B.C.	14,650	17,180	17,040	12,700	
Basal diet B							
plus B vitamins	5	R.B.C.	6,514	5,092	5,650	7,750	None to moderate
No niacin		W.B.C.	20,350	18,630	18,090	14,275	

* x 10^3 = number of red blood cells. **Low nicotinic acid (15 mg./day).

Group 2	No. of pigs		Normals	28 days	48 days	Extent of lesions
Basal diet A	5	R.B.C.	6,812	7,148	6,092	None
All B vitamins		W.B.C.	9,630*	16,200	20,440	
Basal diet A						
All B vitamins	5	R.B.C.	7,236	6,426	5,996	Moderate to extensive
No niacin		W.B.C.	12,340	27,860	27,510	
Basal diet A						
All B vitamins	5	R.B.C.	6,564	7,570	7,334	None to moderate
Tryptophane		W.B.C.	8,120	18,580	19,360	
No niacin						

*Low white blood cell counts, probably due to hog-cholera vaccination two weeks previously.

Group 3	No. of pigs		Normals	32 days	Extent of lesions
Basal diet A					
Tryptophane	5	R.B.C.	7,242	6,382	None to slight*
B vitamins		W.B.C.	21,810	25,660	
Basal diet A					
B vitamins	10	R.B.C.	6,446	6,044	Moderate to extensive (6 pigs)**
No niacin		W.B.C.	22,720	29,807	
No tryptophane					
Basal diet B					
B vitamins	5	R.B.C.	6,600	5,636	Moderate to extensive (2 pigs)**
No tryptophane		W.B.C.	20,270	31,650	
No niacin					

*One pig showed slight lesions. **Balance of lot used in later experiment.

week of the experiment. This was followed, in about a week, by the development of a gray-black diarrhea, which was constant in some cases and intermittent in others. No blood was observed in the feces. Accompanying the development of the intestinal symptoms, there also occurred, in some animals, a mild exfoliative type of dermatitis over the arch of the back and about the ears. There was no evidence of parasitic skin infection. The hair usually appeared rough, and temperatures taken at that time indicated little or no rise above the normal. Chemical analysis of urine indicated a low urinary excretion of N^1-methylnicatinamide. The blood picture, generally, revealed a moderate increase in the number of leucocytes, with a tendency toward a neutrophilia. A slight drop in hemoglobin was noted and red blood cell counts showed a slight decrease in numbers. The relationship between the numbers of red and white blood cells is shown in table 2, demonstrating a tendency for leucocytes to reach a maximum number and erythrocytes to reach a minimum number at about the fifth week, which coincides quite well with the first signs of diarrhea.

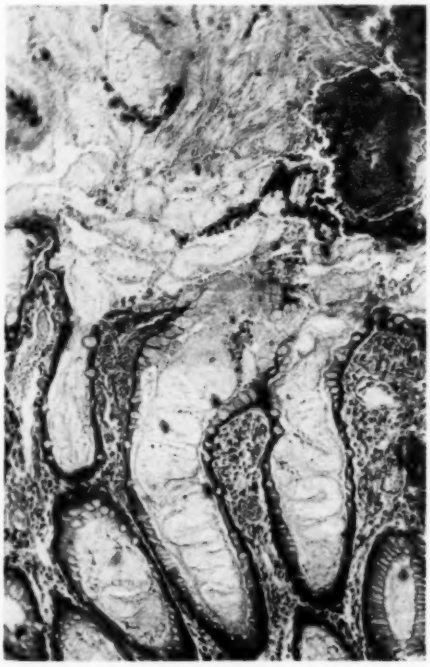

Fig. 2—Section of colon showing mucoid degeneration, some cellular infiltration of the tissue, and an accumulation of neutrophils in the débris. x 100.

Pigs, on experiments lasting as long as ten to twelve weeks, showed a tendency toward increased appetites, disappearance of diarrhea, return of blood components to normal levels, and increased daily gains in weight. Spontaneous recovery of animals reaching a weight of approximately 60 lb. was noted. No incoördination nor paresis of the posterior quarters was observed in these pigs.

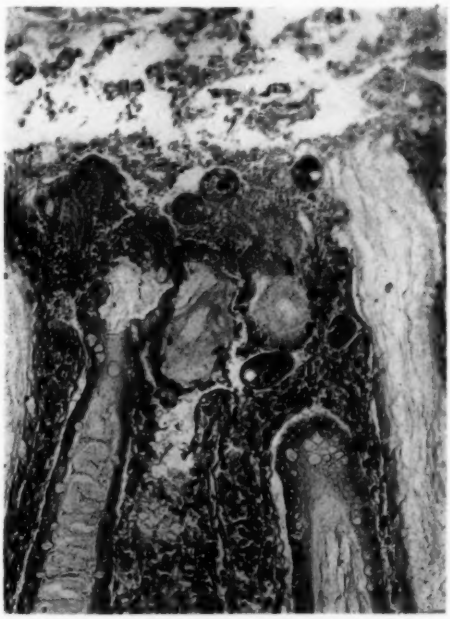

Fig. 3—Section of colon demonstrating the streaming appearance of mucus and invasion of tissue by Balantidium coli. x 100.

Necropsy.—Gross internal pathologic changes were, for the most part, limited to the large intestine. The cecum and as much as 7 ft. of the colon were involved, with masses of feces adhered to the intestinal mucosa by means of tenacious strands of mucus. The size of the adhering masses varied from a few millimeters in diameter to complete coverage of the intestinal wall, for as much as 3 ft. A yellowish exudate occasionally covered portions of the mucous membrane. The wall of the large intestine was usually thickened throughout, slightly doughy in consistency, and showed a tendency to tear easily. There was a mild to moderate hyperemia in all affected sections of the intestine (fig. 1). Animals which

showed a general recovery from symptoms revealed, on autopsy, numerous nodules, many of them containing caseous exudate under the mucosal surface of the rectum

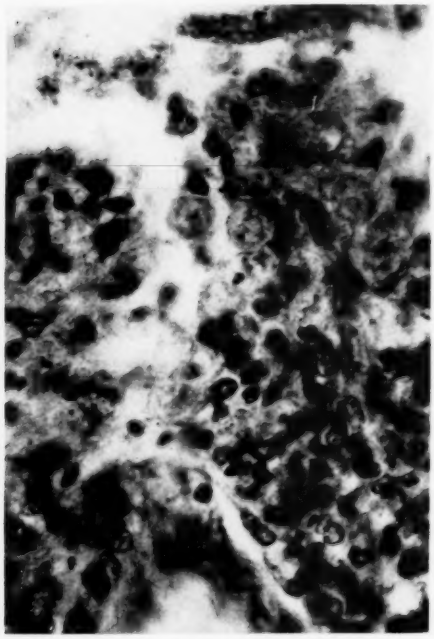

Fig. 4—Section of colon revealing infiltration of the mucosa by lymphocytes and macrophages. x 550.

and caudal portions of the colon. The presence of smooth fibrous areas, typical of tissue regeneration, indicated healed lesions. Mesenteric lymph nodes were enlarged and moderately congested. The stomach and small intestine, in no case, appeared to be involved.

Buccal lesions were not observed in these experiments. However, in later experiments, a few cases of ulcerative stomatitis, in the region of the canine teeth, developed, with fistulous tracts extending dorsally along the lateral surface of the maxilla to necrotic areas in the soft tissues of the snout. Inasmuch as these lesions, which were characteristic of a form of chronic rhinitis ("bull nose"), were found in control pigs, as well as other test animals, no significance was attached to their presence in the niacin-deficient group.

Skin lesions were of a mild exfoliative type of dermatitis, located about the ears and on the arch of the back, and characterized by a scaly epidermis, slight thickening of the skin, and rough coat.

Histopathology.—A histologic study was made of 120 sections taken from the colons of niacin-deficient, treated, and normal control swine. A pathologic condition, characterized by an extensive mucoid degeneration, appeared to be constant in all lesions obtained from swine of the deficiency group. This degeneration, in which all goblet cells were abnormally distended with secretory fluid, was evident in areas where fecal masses had been shown to be adhered.

Fig. 5—Section of colon with tissue invasion by Balantidium coli and increased numbers of macrophages and lymphocytes. x 720.

The lumens of the crypts of Lieberkühn were markedly enlarged, as the result of the increased flow of mucus (fig. 2). This mucus had a very viscous appearance, with thread or fibrin-like-staining characteristics which made it appear to be flowing in streams from the affected crypts (fig. 3). Frequently, some goblet cells extruded an apparently condensed spherical blue-staining (hematoxylin) mass of mucus, which retained its form while being carried along in the mucus flow. This viscous glandular secretion, including some inflammatory cells, appeared to have accumulated about

the orifice of the crypts, enmeshing fecal débris until a relatively large mass adhered to the mucosa.

In many instances, macrophages and

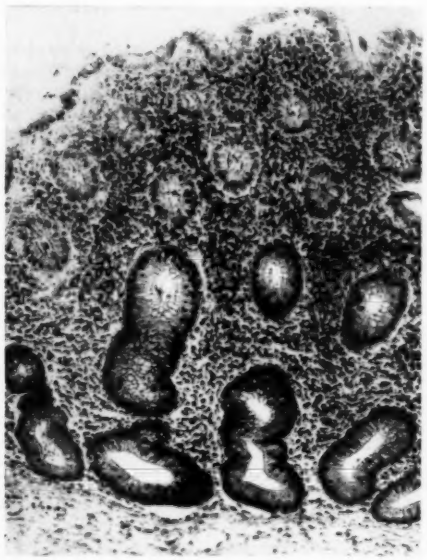

Fig. 6—Section of colon undergoing regeneration, with large numbers of cells in interstitial tissue. x 100.

lymphocytes were the only inflammatory cells present in significant numbers (fig. 4 and 5) but, frequently, neutrophilic infiltration was observed, usually at the periphery of the fecal mass. Such areas, when present, showed evidence of bacterial invasion of the tissues. Since cultures from the affected tissues and intestinal contents of these swine were negative for *S. choleraesuis*, the bacteria seen here were believed to be of the more common enteric group. In some cases, areas showing numerous fibroblasts and lymphocytes were found associated with a regenerating epithelial surface (fig. 6 and 7). Nodules, observed upon necropsy in the colon and rectum of recovered animals, were revealed to be invaginations of the mucosa into the submucosa, forming cystlike cavities surrounded by lymphoid tissue. Normally containing mucus, these cystlike glands become filled with a caseous exudate which they retain long after the remainder of the intestine returned to normal, giving the gross appearance of a nodular abscess, similar to some produced by parasites but showing no calcification.

Areas under the adhered fecal masses showed moderate necrosis, particularly where the tips of the villi were in closer contact with the irritating débris (fig. 3). Around the edges of these areas, under the protective border of the adherent feces, however, bacteria, neutrophils, and a more extensive necrosis were in evidence in many cases, often involving large surfaces.

Hyperemia was almost a constant factor, although the severity depended upon the stage or type of lesion present. Mucoid degeneration was accompanied by mild to moderate hyperemia, while, in severe necrosis with bacterial invasion and neutrophilic infiltration, a relatively more intense hyperemia was noted. Usually areas showing regeneration would also reveal a comparatively high degree of congestion.

Balantidium coli were demonstrated in numbers in the lumen and in the mucosal tissue in one series of experiments (fig. 3 and 5), but conspicuously absent in another. There was little difference in the degree of tissue reaction, but this may have been due to the fact that bacterial invasion was greater in that series showing few Balantidium organisms.

DISCUSSION

From the study of the sections, it seemed evident that the disease associated with

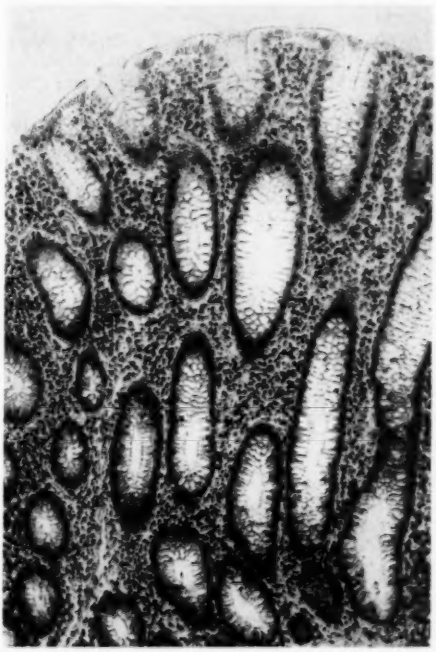

Fig. 7—Section of colon from a recovered pig, showing a normally functioning mucosa. x 100.

niacin deficiency begins with the increased activity of the goblet cells, resulting in an accelerated flow of mucus. The mechanism of this initial stimulus is not known, but reasonably may be other than bacterial since, in many instances, few or no bacteria can be demonstrated in or near these active glands. Also, the organism most often incriminated in swine enteritis, *S. choleraesuis*, was eliminated by culturing affected tissues and intestinal contents. Likewise, in the areas of active mucoid degeneration, there was an absence of neutrophils. However, it is recognized that all bacterial invasions do not promote a neutrophilic reaction. Again, in those areas where bacteria were seen, primarily about the periphery of the fecal mass, there was a tendency towards decreased goblet cell activity and increased neutrophil infiltration.

The fact that animals showing the poorest gains and the most severe necrosis on postmortem were those in which there was also considerable secondary invasion, would lead one to believe that the mucoid reaction of the membrane, over a period of time, may weaken the resistance of the cells and provide a fertile field for secondary invasion by pathogens or facultative pathogens present in the intestine. Both intestinal bacteria and *B. coli* appeared to be complicating factors.

Recovery, in the absence of complications, seemed to be relatively easily achieved by supplying niacin or tryptophane,[7,8] and even occurred spontaneously when the pigs reached a weight of approximately 60 lb. Cases showing heavy secondary infections, however, did not respond as readily to dietary treatment.

SUMMARY

1) Niacin deficiency in swine was characterized by severe mucoid degeneration of the mucosa of the cecum and colon.

2) Grossly, the degenerated areas were recognized by the fecal masses adherent to the mucosal surface by tenacious mucous strands.

3) Cellular reactions involved macrophagic, lymphocytic, and neutrophilic infiltration.

4) Bacteria and neutrophils were most frequently seen about the periphery of the fecal masses.

5) Necrosis was evident in most areas of mucoid degeneration but was most marked where associated with invading organisms.

6) The blood picture revealed a mild to moderate leucocytosis, with some evidence to indicate a slight decrease in erythrocytes.

7) Abscessed submucosal glands characterized the late stages of healing.

References

[1]Birch, T. W., Chick, H., and Martin, C. J.: CCLV. Experiments with Pigs on a Pellagra-Producing Diet. Biochem. J., *31*, (1937):2065-2079.

[2]Braude, R., Kon, S. K., and White, E. G.: Observations on the Nicotinic Acid Requirements of Pigs. Biochem. J., *40*, (1946):843-855.

[3]Chick, H., and Macrae, T. F.: III. Curative Action of Nicotinic Acid on Pigs Suffering from the Effects of a Diet Consisting Largely of Maize. Biochem. J., *32*, (1938):10-12.

[4]Davis, G. K., Freeman, V. A., and Madsen, L. L.: The Relation of Nutrition to the Development of Necrotic Enteritis in Swine. Michigan Agric. Exper. Sta., Tech. Bull., *170*, (1940):1-23.

[5]Goldberger, J., Wheeler, G. A., and Lillie, R. D.: A Further Study of Butter, Fresh Beef, and Yeast as Pellagra Preventives, with Consideration of the Relation of Factor P-P of Pellagra (and Black Tongue of Dogs) to Vitamin B. U. S. Public Health Rep., *41*, (1926):297-314.

[6]Hughes, E. H.: The Minimum Requirement of Nicotinic Acid for the Growing Pig. J. Anim. Sci., *2*, (1943):23-26.

[7]Luecke, R. W., McMillen, W. N., Thorp, F., Jr., and Tull, C.: The Relationship of Nicotinic Acid, Tryptophane and Protein in the Nutrition of the Pig. J. Nutr., *33*, (1947):251-262.

[8]Luecke, R. W., McMillen, W. N., and Thorp, F., Jr.: Further Studies on the Relationship of Nicotinic Acid, Tryptophane and Protein in the Nutrition of the Pig. J. Nutr., *36*, (1948):417-424.

[9]McEwen, A. D.: Necrotic Enteritis of Young Pigs not Associated with Salmonella Infections. Vet. Rec., *49*, (1937):1507-1509.

[10]Miller, D. K., and Rhoads, C. P.: The Experimental Production of Loss of Hematopoietic Elements of the Gastric Secretion and of the Liver in Swine with Achlorhydria and Anemia. J. Clin. Investigations, *14*, (1935):153-172.

[11]Powick, W. C., Ellis, N. R., Madsen, L. L., and Dale, C. N.: Nicotinic Acid Deficiency and Nicotinic Acid Requirements of Young Pigs on a Purified Diet. J. Anim. Sci., *6*, (1947):310-324.

[12]Wintrobe, M. M., Stein, H. J., Follis, R. H., Jr., and Humphreys, S.: Nicotinic Acid and the Level of Protein Intake in the Nutrition of the Pig. J. Nutr., *30*, (1945):395-412.

[13]Worden, A. N., and Slavin, G.: Nicotinic Acid Deficiency in the Pig and Guinea Pig. 1.—The Effects of a "Pellagra-Producing" Diet Upon These Species. J. Comp. Path. and Therap., *54*, (1944):77-87.

The Cytoplasmic Drop and the Cytoplasmic Cap in the Development of Boar Spermatozoa

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A CAREFUL study of the semen picture is of fundamental importance in the diagnosis of male sterility and in the assay of potential male fertility. Various investigators in the field of reproductive physiology have reported the occurrence of several types of abnormal sperm and certain types of immature sperm in semen from some species of animals. One such type of immature sperm is that having a cytoplasmic drop. Rodolfo[8] reported the occurrence of sperm with drops in the semen of boars experiencing even moderate sexual activity. Lagerlof[4] reported that a large number of these immature sperm in bulls occurs both at the beginning of degeneration of spermatogenic epithelium and in a regeneration of the same. Gunn *et al.*[3] agreed with Lagerlof and reported the finding of spermatozoa with drops in sections of the rete testis, in the epididymis, and in the ejaculated semen from abnormal rams. More recently, Rao and Hart[7] and Rao and Berry,[6] working on the development of the spermatozoa of the bull, ram, buck, and Barbary sheep, correlated the descent of the cytoplasmic drop with the physiologic maturity of the sperm, as expressed by their ability for active movement.

McKenzie, Miller, and Bauguess[5] reported the finding of sperm with a cytoplasmic cap in the semen of boars subjected to excessive sexual strain and considered such sperm to be immature. Green[2] described the presence of a vesicular structure at the anterior end of the ejaculated ram sperm. Baylor, Nalbandov, and Clark,[1] with the aid of the electron microscope, were able to find the cytoplasmic cap on ejaculated bull sperm. Rao and Hart[7] and Rao and Berry[6] reported the finding of this cytoplasmic cap on the ejaculated sperm, as well as on sperm from the testes and epididymides obtained from the bull, ram, buck, and Barbary sheep. They observed that the cap underwent partial regression, as the sperm moved away from the testis towards the periphery, but could be noticed on most of the ejaculated sperm.

This work was undertaken with the view of learning the role of the cytoplasmic drop and the cytoplasmic cap in the physiology of the boar sperm and its relation to sperm maturation. It was also desired to study the physiologic changes that sperm undergo as they change in morphology during their passage through the male reproductive tract.

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Materials and Methods

The material was obtained from 4 sexually mature, healthy boars by castration. Their age ranged from 1½ to 5 years, 2 of them being Duroc-Jersey and the other 2, Poland China boars. No attention was paid to their breeding records. In each case, material was examined within one-half to one hour after removal. When further examination was required, the testes were stored in the refrigerator at 45 F. In the boar, the epididymal duct is very prominent, even in the body of the epididymis, and pure epididymal fluid can be obtained from this region by tightly compressing a small portion in the jaws of an artery forceps and incising the ducts lengthwise with a razor blade. The technique described by Rao and Berry[6] was used for staining.

Results

Development of the Boar Sperm.—In the testis, the majority of the sperm has the cytoplasmic drop either surrounding the posterior half of the head, or the neck, or the anterior end of the connecting piece. Sperm, having the first two types of drops, possess a filamentous or filiform connecting piece. In such

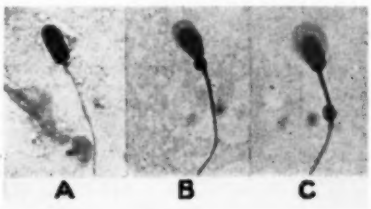

Figure 1

A) Sperm from testis with the cytoplasmic drop at base of head. Note the filamentous or filiform connecting piece. x 750.

B) Sperm from the head of epididymis with the cytoplasmic drop at the anterior end of the connecting piece. Note also the cytoplasmic cap. x 1500.

C) Sperm from tail of epididymis, showing cytoplasmic cap and cytoplasmic drop. x 1500.

sperm, the chief piece of the tail starts as a distinctly thicker portion (fig. 1 [A]). In some cases, the filiform connecting piece also shows some waviness. When the drop is situated at the anterior end of the connecting piece, this region appears normal in thickness and development, but is tortuous in appearance. Most of the sperm in the testis have a pronounced cytoplasmic cap completely surrounding the acrosome and extending laterally to about the middle of the sperm head.

In the head of the epididymis, all sperm present a well-developed cytoplasmic cap, which can readily be demonstrated (fig.1[B]). Also in this region, a large proportion of the sperm have a compact cytoplasmic drop at the anterior end of the connecting piece (fig. 1[C]). The drop may also occur at the posterior end of the connecting piece or at various levels on the connecting piece. The sperm in the head of the epididymis usually have a connecting piece normal in appearance and development, but, at times, some sperm with the drop at the anterior end also show a tortuous connecting piece. The proportion of the various positions of drops on sperm found within this region are given in table 1. The term anterior drop is used to designate any cytoplasmic drop which is situated anterior to the middle of the connecting piece of the sperm, and those drops located posterior to the center are called posterior drops, for the sake of convenience.

In the body of the epididymis, the position of the cytoplasmic drop and the portion of the various types of sperm depend on the particular region from which the material is collected. In the upper third of the body, sperm with anterior drops are more frequent, while in the lower third, sperm with posterior drops are more numerous. The connecting piece is usually normal in thickness and appearance, and most of the sperm in this region have a large cytoplasmic cap.

In the tail of the epididymis, the drop is situated at the posterior end of the connecting piece of most sperm. The proportion of the various types of sperm in this region can be seen from table 1. The cytoplasmic cap is readily recognizable in most sperm (fig. 1[C]).

Although certain types of sperm are more frequent in a particular region of the epididymis, no single type of sperm is restricted to any single region of the reproductive tract. Thus, at times, sperm with posterior drops are most numerous in the head of the epididymis and, similarly, sperm with anterior drops will be more numerous in the tail of the epididymis.

In the ejaculated boar semen, sperm with drops are found more frequently than in the semen from the other species of farm animals. In such sperm, the drop is most commonly found at the posterior end of the connecting piece and sperm with anterior drops are much less frequent. The ejaculated boar sperm has a distinct cytoplasmic cap, which is mostly confined to the anterior end of the head. In the study of the morphology of sperm from the various regions of the male reproductive tract, sperm with bent tails were very rarely found. After storage of either the whole testis or material collected from the tail of the epididymis, the proportion of sperm with bent tails was found to increase when no special precautions for gradual cooling were taken.

TABLE 1—Position of the Cytoplasmic Drop with Reference to the Connecting Piece in Boar Sperm from the Head and Tail of the Epididymis

Boar (No.)	Head of epididymis			Tail of epididymis		
	Anterior drops	Posterior drops	Absent	Anterior drops	Posterior drops	Absent
1	77	23	0	8	62	30
2	81	12	7	10	65	25
3	40	56	4	2	45	53
4	48	48	4	1	72	27

When whole testes were stored in the refrigerator and the sperm from various regions of the testis and the epididymis were examined, it was found that the cytoplasmic drop and the cap disappeared from a varying number of sperm, depending on the length of storage. Similarly, when ejaculated semen or fluid from the tail of the epididymis was stored, undiluted, in the refrigerator, the cytoplasmic cap disappeared during storage.

Development of Motility.—Sperm from the testis of the boar showed very little movement when placed in warm 0.9 per cent saline, the movement being more or less restricted to mere lashings of the tail. Sperm from the head of the epididymis still exhibited only passive movement, while those from the body showed a little more active movement. Material collected from the tail of the epididymis and examined immediately gave a very heavy concentration of nonmotile sperm. When a few of these sperm were transferred to a drop of warm saline, they exhibited active movement. When this epididymal fluid from the tail region was diluted with warm saline and incubated at 100 F. for ten mintues, maximal motility was obtained. (Smears made before and after incubation showed a great reduction in the number of sperm with drops.) The material from the tail of the epididymis was highly viscous, and a similar consistency was pre-

sented by material from the other parts of the epididymis.

Mechanism of Locomotion.—The types of movements exhibited by the boar sperm and the mechanism of locomotion were essentially the same as in the case of sperm of other farm animals. Locomotion was brought about by bilateral lashings of the chief piece of the tail in a horizontal plane, the connecting piece apparently not taking an active part. Changes in direction were brought about by a slight bending of the head about the flexible neck. When motility was maximal, the sperm head always assumed the "on edge" position, remaining almost in a straight line with the connecting piece, and the sperm traveled with the least resistance in a linear fashion. With decline in vigor, the sperm head was found to assume the "on flat" position.

DISCUSSION

The cytoplasmic drop is closely associated with the development of the boar sperm and may be found in different locations on the sperm. In sperm from the testis, the drop was seen surrounding the posterior half of the head, around the neck, or at the anterior end of the connecting piece, these, in all probability, representing successive stages in the descent of the drop. With its descent, the cytoplasmic mass also seems to get organized into a compact cytoplasmic drop. In the epididymis, the sperm from the head region usually have the drop at the anterior end of the connecting piece, while those from the tail region commonly have it at the posterior end. The descent of the drop does not seem to be controlled by the epididymal secretions, owing to the fact that no single type of sperm is limited to any particular region of the reproductive tract. It appears that sperm with anterior drops cannot rid themselves of the drop by active movement because of its being more firmly adherent when located in this region. However, active movement is sufficient to cause the extrusion of posterior drops.[6,7] It seems probable that the cytoplasmic drop has completed its function and is ready to be cast off when it reaches the posterior end of the connecting piece. It is likely that, if sperm in the tail of the epididymis were not ejaculated, the posterior drops might be extruded even before active motility could be manifested by the sperm. This might explain the increasing frequency of sperm without cytoplasmic drops as they move from the head to the tail of the epididymis. There is also evidence to indicate that the cytoplasmic drop becomes smaller and its attachment to the connecting piece gets looser during its downward descent.

Two types of sperm, which have an abnormal connecting piece, were frequently found in the testis. In one case, the connecting piece was filiform and, in the other, it was tortuous. These sperm were usually restricted to the testis only. While the sperm with the filiform connecting piece had the drop either surrounding the posterior half of the sperm head or the neck, those sperm with the tortuous connecting piece had the drop at the anterior end of the connecting piece. These sperm represent immature sperm, probably in successive stages of development. This evidence suggests that the cytoplasmic drop probably has a nutritive function.[7]

The cytoplasmic cap is presented in a very exaggerated form in the boar sperm and is more prominent than in sperm from other species of farm animals. It is, however, most pronounced in the sperm from the deeper regions of the reproductive tract, when the cap is seen to extend laterally to about the middle of the sperm head. In ejaculated semen, the cap is readily seen on most sperm at the anterior end of the head. It is, thus, obvious that the cytoplasmic cap is part of the normal structure of the sperm, and its presence should not be regarded as indicative of the immaturity of the sperm. The cytoplasmic cap probably represents the cytoplasmic remnant of the spermatid, specialized for some specific function, and might be the source of hyalurodinase.

It is also noticed that both the cytoplasmic drop and the cytoplasmic cap disappeared during storage when whole testes were stored for one to two weeks in the refrigerator. The cap also disappeared from sperm from the tail of the epididymis, when epididymal fluid was stored.

The boar sperm from the testis and upper region of the epididymis exhibited movement when diluted with warm saline, but maximal motility was not manifested until they reached the tail of the epididymis. In the fresh epididymal fluid from the tail region, no movement was exhibited by the sperm. But, when this fluid was diluted with warm normal saline and incubated at 100 F. for ten minutes, maximal motility of sperm was evident. It appeared that boar sperm from the testis and the head of epididymis, although capable of locomotion, were unable to exhibit this property, owing to the high concentration of sperm and the viscosity of the natural secretions. However, even when given the proper environment for manifestation of active movement, the sperm from the deeper regions of the reproductive tract were incapable of active motility, probably owing to their physiologic immaturity. When sperm from the tail of the epididymis were given the

same favorable conditions, they underwent a transition from an immotile state to one of maximal motility, presenting evidence to the fact that they were physiologically mature and that the cytoplasmic drop was readily cast off by active motility of the sperm. It is, thus, obvious that sperm with posterior drops were not immature sperm.

The accessory secretions in the boar serve to lower the seminal viscosity and to dilute and disperse the sperm at the time of ejaculation. Although it has been demonstrated[5] that the accessory glands are not necessary for fertility of the boar sperm, the issue, in this species, is complicated by the fact that the urethral glands are very well developed and capable of secreting large amounts of fluid material. This serves to lower the viscosity and dilute the sperm, and, thus, initiate sperm motility, which is probably all that is necessary to enable the sperm to move up the female reproductive tract. Viscosity and high sperm concentration are important factors in the inhibition of sperm motility in the tail of the epididymis in the bull and the boar. The dynamic role of accessory secretions in the initiation of sperm movement is questionable, as this can be achieved by any other suitable diluent.

SUMMARY

The several morphologic types of sperm found in the different regions of the reproductive tract of the boar have been described and their frequency in those areas has been presented. The position of the drop can be taken as indicative of the stage of maturity of sperm, and sperm with drops at the posterior end of the connecting piece are not immature. It appears that the cytoplasmic drop serves a nutritive function.

The cytoplasmic cap is a normal characteristic of all sperm. Although it is present in an exaggerated form on sperm from the deeper regions of the reproductive tract, it can readily be seen on most of the ejaculated sperm and is probably involved in the secretion of hyalurodinase.

Motility of sperm is a very good characteristic which serves to indicate the physiologic maturity of sperm. Sperm from the testis and the head of the epididymis are incapable of active movement even when given optimal conditions, probably due to their physiologic immaturity. However, the nonmotile sperm from the tail of the epididymis go into a state of maximal motility in a few minutes after lowering the viscosity and sperm concentration. Sperm evidence an increasing capacity for active movement with the descent of the drop. The accessory secretions stimulate sperm movement in this species by eliminating the factors which suppress sperm motility—high viscosity and high sperm concentration.

References

[1]Baylor, M. B., Nalbandov, A., and Clark, G. A.: Electron Microscope Study of Sperm. Proc. Soc. Exptl. Biol. and Med., 54, (1943):229-232.

[2]Green, W. W.: The Chemistry and Cytology of the Sperm Membrane of Sheep. Anat. Rec., 76, (1940):455-473.

[3]Gunn, R. M. C., Sanders, R. N., and Grange, W.: Studies in Fertility in Sheep. Bull. Council Sci. Indust. Res., Australia, 148, (1942):1-140.

[4]Lagerlof, N.: Sterility in Bulls. Vet. Rec., 48, (1936):1159-1171.

[5]McKenzie, F. F., Miller, J. C., and Bauguess, A. C.: The Reproductive Organs and Semen of the Boar. Missouri Agric. Exper. Sta., Res. Bull. 279, 1938.

[6]Rao, C. K., and Berry, R. O.: Observations on the Cytoplasmic Drop and Cytoplasmic Cap in Sperm Development in Domestic Sheep, Domestic Goats and Barbary Sheep. (*Ammotragus Lervia*). In press.

[7]Rao, C. K., and Hart, G. H.: Morphology of Bovine Spermatozoa. Am. J. Vet. Res., 9, (1948): 286-290.

[8]Rodolfo, A.: Morphology of Spermatozoa. Philippine J. Sc., 55, (1934):165-173.

Preliminary Observations in the Comparative Prophylactic Vaccination of Dogs Against Rabies with Living Virus Vaccines and Phenolized Vaccine

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IN THE COURSE of the work on adaptation of four strains of rabies virus to the developing chicken embryo,[1] it became apparent that the peripherally administered Flury egg-adapted strain[2] was avirulent and of markedly modified virulence when inoculated intracerebrally in rabbits.[1] Moreover, the same strain at 22 and 75 egg-passage levels, when injected into the masseter muscles of dogs, did not seem to engender any signs of illness, and the tissues from these animals, obtained at various intervals after inoculation, seemed to be devoid of any infective power.[3] In view of the above observations, a more comprehensive study was outlined in which the immunizing potency of the egg-adapted Flury strain was compared with another chicken embryo-adapted and a rabbit-brain-fixed strain of living virus, as well as with a vaccine prepared from inactivated virus. The results of these experiments are the subject of the present report.

MATERIALS AND METHODS

Strains of Rabies Virus.—(a) Flury Strain.—The history of this strain has been given in a previous report.[1] The virus had undergone 138 passages in chick brain, followed by 40 passages in the developing chicken embryo.

b) NIH Strain.—This is a mouse-adapted fixed strain of rabies virus, obtained from the National Institutes of Health, Bethesda, Md., for routine use as a standard challenge virus in the vaccine mouse potency test required by the Biologics Control Laboratory.[4] This strain had been adapted to the developing chicken embryo and then passaged through 26 egg generations. Both the Flury strain and the NIH strain were used in the form of a lyophilized preparation of infected chicken-embryo tissue.

c) P.M. Strain (Pitman-Moore No. 980).—A rabbit-fixed strain of virus, obtained through the kindness of Dr. John T. Wright of the Biologics Control Laboratory, National Institutes of Health, Bethesda, was passed in rabbits and used as a lyophilized suspension of rabbit-brain tissue.

d) Lederle Strain.—This is a strain of fixed rabies virus brought by Dr. Anna Williams from the Pasteur Institute, Paris, to the laboratories of the New York City Department of Health. The strain, obtained in 1913 by the Lederle Laboratories from the New York City Department of Health, has been maintained in rabbit-brain tissue in these laboratories. In the present experiments, the strain was used in the form of phenolized vaccine prepared from horse brain.

e) NYC Strain.—Through the kindness of Miss Virginia Guevin of the Bureau of Laboratories, New York City Department of Health, a submaxillary salivary gland of a rabid dog was obtained, and suspensions were passed through three passages in the salivary glands of dogs in these laboratories. In the present experiments, it was used in the form of a frozen suspension of canine submaxillary salivary gland as challenge virus for the vaccinated and control dogs.

f).—For the neutralization tests, the "Alabama" strain was used. This fixed strain of rabies virus had been given 557 rabbit-brain passages in the production of virus for preparing the Semple type of vaccine by the laboratories of the Alabama State Board of Health. After one additional rabbit passage, this strain was kept in the form of frozen suspension of rabbit-brain tissue.

Animals.—Mongrel dogs of both sexes, between 6 to 51 months of age (average 16 months) and weighing from 8 to 42 lb., were kept in large, open-air wire cages, 2 to 3 animals per cage, under close daily clinical observation for two months prior to vaccination and during the entire test period. The dogs were grouped to assure even age distribution among the different test groups.

Dead dogs were carefully autopsied and the brain and submaxillary gland tissues removed under aseptic precautions. Impression smears

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of Ammon's horn, cerebral cortex, and cerebellum were made and stained with Sellers' stain for detection of Negri bodies. In addition, 10 per cent suspensions of the brain and salivary gland tissues in physiologic saline solution were subinoculated intracerebrally into groups of 6 mice each.

The mice were observed for twenty-one days for any signs of illness. Serial tenfold dilutions of the Flury, NIH, P.M., and NYC strains of rabies virus in 10 per cent normal rabbit-serum saline were titrated intracerebrally in Swiss albino mice 21 to 28 days of age, 6 mice being used for each dilution. The l.d.$_{50}$ titers were calculated according to the Reed and Muench formula. Swiss albino mice of the same age were used for the neutralization tests.

Neutralization Test.—Frozen standardized suspensions of rabbit brains infected with fixed Alabama strain of rabies virus were used in the neutralization test. Dilutions, containing about 200-l.d.$_{50}$ doses of virus, were prepared in 10 per cent normal rabbit-serum saline and aliquots were mixed, respectively, with an equal volume of dog serum, either undiluted or with fourfold dilutions in saline. The serum-virus mixtures were incubated for one hour at 37 C. and refrigerated at 4 C. for one hour, and then injected intracerebrally in 0.03-cc. amounts into Swiss albino mice, using 4 or 6 litter mice for each serum-virus mixture.

The injected mice were observed for twenty-one days and the sick and dead were recorded. In the tables, the results of inoculation for any given group of mice are indicated by survival ratios, in which the numerator represents the number of survivors and the denominator the number inoculated (less those that had died of nonspecific causes). The 50 per cent mortality end point titer was calculated in those cases in which dilutions of respective serums were tested.

EXPERIMENTAL

Vaccination.—After the preliminary observation period of two to three months, the dogs were bled from the radial vein and inoculated with the vaccines in the upper quadrant of the posterior surface of the thigh muscles (musculus semitendinosus and musculus semimembranosus). The dogs were grouped as shown in Table A.

No signs of illness were observed in any of the animals of the four vaccinated groups during the sixty-day observation period between vaccination and challenge inoculation. All of the dogs were bled at twenty-two (bleeding B), forty-three (bleeding C), and sixty days (bleeding D) after vaccination. The postvaccination, as well as the prevaccination, serums were submitted to neutralization tests to determine the time of appearance and duration of circulating rabies antibodies.

Challenge.—Sixty days after vaccination, the dogs were challenged with street virus. Fifteen unvaccinated dogs, kept under the same conditions as the vaccinated animals, were bled from the radial vein and inoculated with the street virus at the same time as the vaccinated dogs received the challenge inoculation. The dogs were inoculated with a total dose of 0.2 cc. of a 10 per cent canine salivary gland suspension, injected in 0.1-cc. amounts into the masseter muscle of each side of the face. The inoculum, injected intracerebrally into mice, had a titer of $10^{-4.30}$ l.d.$_{50}$

The results of the challenge test are summarized in table 1. Of the 15 unvaccinated control animals, 8 died of rabies after the inoculation of the street virus. In each, either Negri bodies were shown to be present in brain-tissue smears or rabies virus was isolated from the tissues, or both. It should be noted that 1 of the control animals died after an unusually long incubation period of 257 days. As all of the animals had been kept strictly isolated during the entire observation period, its death must be ascribed to the original inoculation and not to any contact infection. Moreover, Negri bodies were present in the brain-tissue smears and rabies virus was isolated from

TABLE A

Group	Number of dogs vaccinated	Amount of inoculum	Type of material inoculated	Calculated intracerebral mouse l.d.$_{50}$ contained in each inoculum
1	9	5 cc.	20% chicken-embryo suspension, 40th egg-passage Flury strain living virus, rehydrated from lyophilized state.	$10^{5.30}$
2	8	5 cc.	20% chicken-embryo suspension, 26th egg-passage NIH strain living virus, rehydrated from lyophilized state.	$10^{6.50}$
3	8	5 cc.	2% rabbit-brain suspension, infected with P.M. strain living virus, rehydrated from lyophilized state.	$10^{6.25}$
4	7	5 cc.	20% suspension of phenolized horse-brain tissue, prior to inactivation infected with the Lederle strain of virus.	*

*The results of the mouse potency test indicated that the phenolized vaccine protected mice against 8,790 mouse intracerebral l.d.$_{50}$.

both the brain tissue and from salivary glands.

Among the vaccinated dogs, death due to the challenge inoculation with the street virus was observed in two instances (table 1). In group 2, 1 of the vaccinated dogs succumbed to rabies infection nineteen days after the challenge inoculation, or seventy-nine days after vaccination with the NIH-chicken embryo-adapted strain. In group 4, a dog, which had been vaccinated with the phenolized vaccine, died 173 days after the challenge inoculation, or 233 days after vaccination. In each of these animals, diagnosis of rabies was amply confirmed by positive Negri body smears and by isolation of the virus through subinoculation of brain and salivary gland tissues into mice.

Serologic Studies on Serums of Vaccinated Dogs.—Serum samples obtained from dogs prior to vaccination, and at regular intervals after vaccination until the day before the challenge inoculation, were submitted to neutralization tests against rabies virus. Because of the unusually large number of samples involved, aliquots from infected mouse-brain suspensions were mixed with equal volumes of undiluted dog serum; the mixtures, after incubation and refrigeration, were inoculated intracerebrally in groups of 4 mice each.

The results of the tests are shown in table 2. Because only a single dilution of serum was tested against a single dilution of virus, and because of the small number of mice injected with the serum-virus mixtures, the data presented in table 2 should be considered only as tentative and not definitely indicative of the neutralizing power of a given serum sample. For the analysis of results, the following criterion was adopted: Serums giving 4/4 or 3/4 survival ratio of mice were considered as having definite neutralizing power, those with 0/4 or 1/4 survival ratio were considered as being devoid of neutralizing power, and a 2/4 survival ratio was classified as doubtful, although the serum was considered to possess some neutralizing ability.

Keeping the above tentative classification in mind, analysis of the results with prevaccination of samples of serums of dogs in group 1 indicates that dogs 17 and 19 showed some neutralizing power. The neutralizing ability of the serums from the same 2 dogs seemed to be more pronounced after vaccination. The prevaccination serum samples obtained from the remaining dogs in the same group seemed to show no neutralizing power, or only in negligible degree. Conversely, the neutralizing ability of serums of all of the dogs increased after vaccination, and this level persisted with only minor fluctuations until the time of challenge.

In group 2, only two of the eight prevaccination serums showed some neutralizing power. In this group, the serums obtained twenty-two days after vaccination showed, without exception, rise in neutralizing ability. With the possible exception of serum from dog 16, this increase was still apparent at the forty-third postvaccination bleeding day. However, the neutralization tests performed with serums obtained sixty days after vaccination indicate that the neutralizing antibodies in the blood of dog 16 had dropped to a low level, and the serum sample of dog 21 gave doubtful results. All of the remaining serums still showed definite neutralizing power. It may be of interest to note that in this group of animals, dog 16 was the only one which succumbed to rabies after the challenge inoculation.

In group 3, prevaccination serum of dog 7 showed definite neutralizing power which persisted throughout the prechallenge period. The prevaccination serums of the remaining dogs gave negative results, but became positive after vaccination and without exception remained positive up to the time of the challenge inoculation.

In group 4, the prevaccination serum from dog 34 showed neutralizing power which persisted throughout the observation period. Serums of the remaining dogs were devoid of neutralizing ability prior to vaccination and all but 1 showed a definite and persistent rise in protective power after vaccination. The 1 exception was dog 40 whose twenty-second-day postvaccination serum showed definite rise

TABLE 1—Results of Inoculations with Street Virus in Dogs Vaccinated Against Rabies and in Unvaccinated Control Dogs

Group	Vaccine*	Number of dogs		Challenge			
		Inoculated	Died	Day of death**	Negri bodies	Rabies virus isolated from: Brain tissue	Salivary gland
1	Flury	9	0				
2	NIH	8	1	19	1+	1+	1+
3	P.M.	8	0				
4	Lederle	7	1	173	1+	1+	1+
5	Unvaccinated controls	15	8	14 (2) 16 (2) 19, 25 39, 257	7+ 1—	8+	7+ 1—

*Flury = chicken-embryo tissue, living virus; NIH = chicken-embryo tissue, living virus; P.M. = rabbit-brain tissue, living virus; Lederle = phenolized vaccine.
**From the day of inoculation with street virus.
+ = positive; — = negative.

in neutralizing power but had dropped to a negligible level at the two subsequent bleedings. It is again noteworthy that only this dog in group 4 died of rabies following the challenge inoculation with street virus.

The results of these tests seem to indicate that, regardless of the type of inoculum used, vaccination, with few exceptions, was followed by the appearance of homologous antibodies. However, as cautioned above, the evidence was considered to be of presumptive rather than of definite character, and postvaccination serums from 3 to 4 dogs in each experimental group were submitted to a more sensitive, and therefore more indicative, test than the one given above. In this sensitive test, aliquots of serial fourfold dilutions of respective serums were mixed with equal volumes of a dilution of virus which contained 32 l. d_{50}. The serum-virus mixtures were incubated for one hour at 37 C. and then injected intracerebrally into groups of 6 mice each.

TABLE 2—Summary of Results of Neutralization Tests Against 100 $L.D_{50}$ of Fixed Rabies Virus at Different Intervals from Dogs after Vaccination Against Rabies

GROUP 1: Dogs vaccinated with Flury strain - living virus					GROUP 2: Dogs vaccinated with NIH strain - living virus				
Dog (No.)	Survival Ratio of Mice *				Dog (No.)	Survival Ratio of Mice *			
	A	B	C	D		A	B	C	D
3	0/4	4/4	2/3	4/4	5	0/4	4/4	3/4	4/4
4	0/4	4/4	3/4	3/4	10	3/4	4/4	3/4	3/3
8	1/4	4/4	4/4	4/4	16**	1/4	3/4	2/4	1/3
9	1/4	4/4	3/4	4/4	21	0/4	4/4	3/3	2/4
17	2/4	4/4	4/4	2/4	24	0/4	4/4	4/4	4/4
19	3/4	4/4	4/4	4/4	25	1/4	3/3	3/3	4/4
28	0/4	4/4	4/4	4/4	26	0/4	4/4	4/4	3/4
33	0/4	4/4	4/4	3/4	29	2/4	4/4	4/4	4/4
36	0/4	4/4	3/4	4/4					
GROUP 3: Dogs vaccinated with P.M. strain - living virus					GROUP 4: Dogs vaccinated with phenolized vaccine				
Dog (No.)	Survival Ratio of Mice *				Dog (No.)	Survival Ratio of Mice *			
	A	B	C	D		A	B	C	D
2	1/4	4/4	3/4	4/4	18	0/4	2/4	2/4	3/4
6	0/4	4/4	4/4	4/4	23	1/4	4/4	4/4	3/4
7	4/4	4/4	4/4	3/4	27	0/4	4/4	4/4	3/4
11	0/4	4/4	3/4	3/4	32	0/4	4/4	4/4	4/4
12	1/4	4/4	4/4	4/4	34	4/4	4/4	4/4	4/4
15	0/4	4/4	3/4	2/3	35	0/4	4/4	4/4	4/4
20	0/4	4/4	3/4	4/4	40**	0/4	4/4	1/4	1/4
39	0/4	4/4	4/4	4/4					

*Survival ratio of mice inoculated intracerebrally with virus and serum obtained from dogs bled on the following days:
A = prevaccination; B = twenty-two days after vaccination; C = forty-three days after vaccination; D = sixty days after vaccination and one day before challenge inoculation.
**Died of rabies after challenge inoculation with street strain of rabies virus.

TABLE 3—Summary of Results of the Sensitive Neutralization Test with Serums Obtained from Dogs Vaccinated Against Rabies and from Unvaccinated Control Dogs

GROUP 1
Dogs vaccinated with FLURY strain—living virus

Dog (No.)	Serum*	Survival ratio of mice injected with rabies virus and dilutions of serum 1:2	1:8	1:32	1:128	1:512	1:2048	Minimal protective titer of serum
4	A	0/6	0/6					<1:2
	B		5/6	2/6	0/6	1/6		1:25
	C		5/6	5/6	0/6	2/6	1/6	1:70
	D		4/6	2/6	0/6	0/6	0/6	1:15
8	A	4/6	1/6					1:3
	C	6/6	5/6	2/6	0/6			1:20
19	A	3/5	2/6	0/6	3/6			1:6
	B		6/6	5/6	3/6	0/6		1:105
	C		5/6	3/6	1/6	1/6	1/6	1:50
	D		5/6	3/6	1/6	0/6	2/6	1:50

GROUP 2
Dogs vaccinated with NIH strain—living virus

Dog (No.)	Serum*	Survival ratio of mice injected with rabies virus and dilutions of serum 1:2	1:8	1:32	1:128	1:512	1:2048	Minimal protective titer of serum
5	A	0/6	2/5					<1:2
	B		5/5	6/6	3/6	1/6		1:160
	C		5/6	6/6	0/6	3/6	0/6	1:75
	D		4/6	3/6	0/6	2/6	0/6	1:65
10	A	1/6	0/6	0/6	0/6			<1:2
	B		6/6	6/6	5/6	0/6		1:220
	C		4/5	4/6	2/6	1/6	1/6	1:75
	D		4/5	4/5	3/6	0/6	0/6	1:85
24	A	0/6	0/6					<1:2
	B		5/6	5/6	5/6	3/5		1:315
	C		6/6	4/6	5/6	2/6	0/6	1:330
	D	6/6	6/6	5/5	5/6	1/6		1:255
29	A	2/6	0/6					<1:2
	B		6/6	6/6	4/6	0/6		1:180
	C		5/6	5/6	1/6	0/6	0/6	1:55
	D		6/6	6/6	1/6	0/6	0/6	1:75

GROUP 3
Dogs vaccinated with P.M. strain—living virus

Dog (No.)	Serum*	Survival ratio of mice injected with rabies virus and dilutions of serum 1:2	1:8	1:32	1:128	1:512	1:2048	Minimal protective titer of serum
2	A	1/6	5/6					1:4
	B		6/6	6/6	6/6	4/6		>1:512
	C		6/6	4/6	4/6	0/6		1:130
	D		6/6	5/6	2/6	1/6		1:95
7	A		2/6	1/6	2/6	0/6		1:10
	B		6/6	3/6	1/6	0/6		1:40
	C		6/6	4/6	0/6	0/6		1:45
	D		4/6	2/6	1/6	2/6		1:25
20	A	3/6	0/6					1:2
	B		5/6	3/6	1/6	0/6		1:30
	C		6/6	1/6	0/6	0/6		1:20
	D		5/6	1/6	0/6	0/6		1:15

GROUP 4
Dogs vaccinated with phenolized vaccine

Dog (No.)	Serum*	Survival ratio of mice injected with rabies virus and dilutions of serum 1:2	1:8	1:32	1:128	1:512	1:2048	Minimal protective titer of serum
23	A	5/6	6/6					>1:8
	C	5/6	4/6	3/6	0/6			1:15
	D	6/6	0/6	2/6	0/6	0/6		1:5
34	A		5/6	6/6	4/6	2/6		1:215
	B		6/6	6/6	6/6	5/6		>1:512
	C		6/6	6/6	5/6	5/6		>1:512
	D		6/6	6/6	6/6	4/6		>1:512
40**	A	5/6	0/6					1:3
	B		4/5	1/5	0/6	1/6		1:20
	C	3/6	1/6	0/6	1/6			1:3
	D	4/6	1/6	0/6	1/6			1:4
18	A	0/6	1/6					<1:2
	B	6/6	4/6	5/6	2/6			1:55
	D	5/6	5/6	2/5	3/6	0/6		1:30

GROUP 5
Unvaccinated control dogs

41	E	5/6	2/6	1/6	0/6			1:6
51	E	1/6	0/6					<1:2
54**	E	4/6	1/6					1:3
56	E	5/6	4/6	2/6	0/6			1:15
58	E	4/6	0/6	2/6				1:4
71	E	0/6	0/6					<1:2
72	E		6/6	5/6	4/6			>1:128
73	E	4/6	0/6					1:3

*A = prevaccination bleeding.
B = bled twenty-two days after vaccination.
C = bled forty-three days after vaccination.
D = bled sixty days after vaccination and one day before challenge inoculation.
E = prechallenge bleeding of unvaccinated controls.

**Died of rabies after inoculation with the street virus.

Included in the same test were serums obtained before the inoculation with street virus from the unvaccinated control dogs which had shown no signs of rabies infection. (One exception was dog 54, which came down with rabies 257 days after inoculation with the street virus. *See* table 1.)

The prevaccination serums from dogs in group 1 (*see* table 3) showed very low protective titers, but about a fivefold increase was apparent after vaccination. With minor fluctuations, the rise in titer persisted until the time of challenge (bleeding D) and, as will be recalled, these dogs remained refractory to the challenge inoculation. In group 2, the serologic response after vaccination seemed to be better than in group 1 although, with the exception of dog 24, the titer showed a tendency to decline as indicated by the results of the forty-third-and sixtieth-day bleedings. As already stated, dog 16 had succumbed to challenge with the street virus, and serum from this dog was not included in this test because of the small amount of serum available for the preliminary neutralization test (table 2). In group 3, the response of dog 2 after vaccination was remarkably good, but again a decline in titer was noted on the sixtieth-day postvaccination bleeding. The rise in protective titer in the postvaccination serums of the 2 remaining dogs was much lower than that of dog 2, but comparable to those in group 1. The results of the neutralization tests with serums of dogs in group 4 indicate that 1 of the animals (dog 34) possessed, as compared to the other dogs, a high level of circulating antibodies prior to vaccination and that the vaccination boosted the protective titer to a high level, which persisted up to the time of challenge. The prevaccination titer of serum of dog 23 was found to be higher than 1 : 8, but postvaccination samples from the same animal gave low titers, indicating that probably the prevaccination titer was near its end point. The serum of dog 40 showed some increase in its protective titer at the twenty-second-day bleeding but, at the time of the two subsequent bleedings, the titer had dropped to its original low level and this dog succumbed to rabies (table 1).

The neutralization tests with serum samples from the 8 unvaccinated dogs in group 5, obtained prior to the inoculation with the street virus, indicate that the protective titer of serum of dog 72 was high enough to consider this animal as possibly being immune. This may also apply to dog 56, although with much more reservation since the titer was only 1 : 15. Serums of the remaining 6 dogs in the same group show only negligible or no protective power, and yet, of these 6, only dog 54 succumbed to rabies eight and one-half months after inoculation.

DISCUSSION

The experimental data presented in the foregoing should be considered as of preliminary nature. First, the number of animals in each experimental group was statistically not significant. Second, the unvaccinated control dogs showed a considerable degree of resistance to rabies infection, as only 53 per cent died of rabies after the inoculation with street virus. In 2 of the 7 control animals which remained refractory to rabies infection, the resistance was indicated by the presence of circulating rabies antibodies. In the remaining 5, the results of the neutralization test on their serums were essentially negative (table 3); yet the animals did not show any signs of disease during the entire observation period. In a few instances, the results of the neutralization tests on prevaccination serum samples indicated a preëxisting level of immunity; how many of the remaining dogs, serums of which did not show any neutralizing power (table 1), should also be included in the category of resistant or immune animals is hard to say. However, to be objective, one should make allowance for at least 2 or 3 animals in each of the vaccinated groups which probably were resistant to rabies infection, irrespective of vaccination.

Taking the above observations into consideration in the analysis of the experimental data, one may conclude that the vaccines, taken as a whole, showed a good level of immunizing potency. Since preliminary observations indicated that the Flury strain, chicken-embryo virus vaccine and the P. M. strain, rabbit-brain virus vaccine may be capable of exhibiting antigenic qualities approximately equal to, if not better than, the other vaccines, it is felt that further study is warranted to ascertain possible differences in the immunizing power of any of the vaccines used.

The preliminary neutralization tests summarized in table 2 show that practically all of the animals, irrespective of the vaccine used, reacted with formation of antibodies by the twenty-second day after vaccination, and, in the majority of the animals, these antibodies were still present in the serums drawn forty-three and sixty days after vaccination. In a few of the dogs, the neutralizing power seemed to decrease, and it was among these animals that the 2 vaccinated dogs succumbed to the challenge inoculation. The results of the highly sensitive type of neutralization test summarized in table 3 appear to confirm the approximately equal antibody-eliciting power of the vaccines tested. The protective titers of the serums seemed to be slightly higher in the

twenty-second-day postvaccination bleeding than at the subsequent bleeding dates. However, in view of the results obtained in the neutralization tests on serums of the unvaccinated dogs, it is difficult to correlate the serologic evidence and the resistance of the animal to experimental infection.

The conclusion one may draw is that, of the four types of vaccines tested, two protected all of the animals against challenge inoculation with street virus. Of these two, because of the markedly reduced virulence for experimental animals and dogs,[1, 3] the vaccine prepared from chicken-embryo tissue infected with the Flury egg-adapted strain of rabies virus may offer, from the standpoint of safety of the vaccination procedure, advantages over the vaccine prepared from rabbit-brain-fixed strain of virus.

SUMMARY

Dogs were vaccinated with a single, 5-cc., intramuscular injection of chicken-embryo suspension infected with egg-adapted strains of living virus, with rabbit-brain suspensions infected with rabbit-brain-fixed strain of living virus, and with phenolized vaccine, respectively.

Sixty days after vaccination, the 32 vaccinated animals and 15 unvaccinated control dogs were given intramasseter inoculation of a canine salivary gland suspension infected with street virus. Eight of the control animals and 2 of the vaccinated succumbed to rabies infection after inoculation with the street virus.

There were no rabies deaths in two of the vaccinated groups: the group vaccinated with egg-adapted Flury strain, which was previously found to be attenuated for dogs, and the group vaccinated with rabbit-brain-fixed virus.

Appearance of homologous antibodies was elicited in serums of practically all of the vaccinated animals regardless of the type of vaccine employed. In a few of the dogs, the decrease in antibody level ran parallel to the length of time after vaccination. However, of the 7 control dogs which resisted the inoculation with street virus, serums of 5 animals seemed to show either very low level, or no, neutralizing antibodies.

References

[1]Koprowski, H., and Cox, H. R.: Studies on Chick Embryo Adapted Rabies Virus. I. Cultivation and Pathogenic Properties. J. Immunol., *60*, (1948):533-554.

[2]Leach, C. N., and Johnson, H. N.: Human Rabies, With Special Reference to Virus Distribution and Titer. Am. J. Trop. Med., *20*, (1940): 335-340.

[3]Koprowski, H.: Data to be published.

[4]Federal Security Agency, Public Health Service, National Institutes of Health, Bethesda, Md. Minimum Requirements: Rabies Vaccine. 2nd revision, Oct. 1, 1946.

The Failure to Recover Newcastle Virus by Egg Inoculation of Dilutions of Organ Suspensions

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THE ROUTINE procedure for the isolation of Newcastle virus in this laboratory consists in emulsifying a small quantity of tissue in about 1.5 cc. of broth and inoculating each of three or more eggs into the allantoic sac with a dose of 0.2 cc., according to a procedure which has been previously described in detail.[1]

It is well known that the virus of Newcastle disease may not be demonstrated in every tissue of a bird submitted for routine diagnosis, nor in any tissue of some birds whose mates have yielded the virus.

The presence of the virus in the brain, associated with nervous symptoms, and its absence from the spleen or other internal organs might be interpreted as a case in the late stage of the disease with autosterilization of the internal organs. This is exemplified by bird 1 of case 25584 in table 1. However, the failure of this explanation is evident in the results on bird 2 of case 25670 in table 1. This chicken showed paralysis, and, although the virus was recovered from the spleen, it was absent from the brain. Again, bird 2 of case 25640 showed no nervous symptoms, but brain, yolk, and respiratory exudate yielded the virus, and bird 2 of case 25659, likewise, exhibited no nervous symptoms, but virus was recovered from brain, spleen, and yolk.

TABLE 1—Results of Routine Egg Inoculations of Suspensions of Organs and Exudate from Chicken to Select Tissues for Inoculation After Further Dilution

Case No. and date	Bird No. and age	Symptoms and/or autopsy	Date of examination		Suspension examined				
			Tissue	Mucus	B*	S	Y	M-F	M-PS
25584 12/6/47	1—pullet	Nervous symptoms Air sacs clouded Flaccid ova	12/30	3/23	+	—	0	—	—
	2—pullet	Air sacs clouded Flaccid ova	12/30	3/23	—	—	0	—	—
	3—pullet	Liver enlarged and infiltrated		3/23	0	0	0	+	+
25670 1/5/48	1-3 wk. chicken	Paralysis Clouded air sacs	1/7		—	—	0	0	0
	2-4 wk. chicken	Paralysis Clouded and thickened air sacs Tracheal exudate	1/7		—	+	0	0	0
	3—hen	Paralysis Clouded air sacs Atretic ova	1/7		—	—	0	0	0
25640 1/6/48	1—pullet	Clouded air sacs Flaccid ova Yolk in abdominal cavity	1/9	3/26	—	—	—	—	—
	2—pullet	Clouded air sacs Flaccid ova Yolk in abdominal cavity	1/9	3/26	+	—	+	—	+
25659 1/8/48	1—pullet	Mucus in trachea Air sacs clouded Flaccid ova	1/9	3/26	—	—	—	—	—
	2—pullet	Mucus in trachea Air sacs clouded Flaccid ova	1/9	3/26	+	+	+	—	—

*B, S, and Y refer to brain, spleen, and yolk, respectively; M-F and M-PS refer to respiratory exudate rendered bacteriologically sterile by filtration (Boerner filters) and by treatment with a mixture of penicillin and streptomycin; + means that virus was isolated; — that it was not recovered; and 0 that no examination was made.

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Failure to recover the virus from any tissue of some birds whose mates had yielded virus might be explained by assuming that these birds were in the late stages of the disease and that immune bodies had developed sufficiently to mask the presence of the virus. The possibility of recovering virus from such tissues by injecting eggs with more dilute suspensions presented itself. By such a procedure, it was hoped that a dilution might be reached that was beyond the limit of activity of the immune bodies and yet within the range of activity of the virus. Todd[2] has already demonstrated that a neutral mixture of fowl-plague virus and its immune serum, as determined by intramuscular inoculation, will produce plague if diluted from fourfold to tenfold *in vitro* before inoculation. Likewise, a neutral mixture was shown to produce plague when injected intravenously, in which case dilution was effected *in vivo*.

To determine whether dilution of tissue suspensions would result in a release of virus, suspensions that had given negative results on routine inoculation were selected from birds whose mates had yielded virus. Thus, in case 25584, the diagnosis of Newcastle disease was established by recovery of the virus from the brain of bird 1 and from the respiratory exudate of bird 3, but brain, spleen, and respiratory exudates of bird 2 were not found to contain virus, as determined by the routine procedure. Therefore, the original brain and spleen emulsions of bird 2 were diluted from 1 : 50 to 1 : 1,500, and each dilution was inoculated into the allantoic sac of 3 or more 10- or 11-day embryonating eggs. In each of the remaining 3 cases selected for study, a tissue or tissues from 1 bird yielded the virus to establish the diagnosis and in each case 1 or more birds failed to yield tissues that were positive by the routine procedure. In addition, two tissues that were negative on routine examination were from birds that yielded one or more positive tissues. The results of these primary inoculations are given in table 1.

It might appear that the dilutions tested were not great enough, in view of the fact that the virus is often active in a dilution of 10^{-8} or 10^{-9}, but, since the inoculation is made into the allantoic fluid, a further dilution is thereby effected. The volume of allantoic fluid in a 10- or 11-day embryonating egg is roughly about 6 cc., and, therefore, the 0.2 cc.-dose of inoculum routinely used would undergo a dilution of about 30 times. Therefore, an inoculum diluted 1:1,500 actually becomes about 1:50,000.

The results obtained from the inoculation of more dilute suspensions are given in table 2. These results show that dilution did not change the original result.

TABLE 2—Results of Egg Inoculation of Various Dilutions of Tissues

Case No. and date	Date tested	Tissue and bird	Dilution tested: Non dil.	1:50	1:100	1:500	1:1,000	1:1,500
25584 12/6/47	3/ 2/48	B 2*	—	—	—	—	—	—
		S 2	—	—	—	—	—	—
25670 1/5/48	5/14/48	B 1	—	—	—	—	—	0
		S 1	—	—	—	—	—	0
	5/ 7/48	B 2	—	—	—	—	—	—
		B 3	0	—	—	—	—	0
		S 3	0	—	—	—	—	—
25640 1/6/48	3/ 2/48	B 1	—	—	—	—	—	—
		S 1	—	—	—	—	—	—
		Y 1	—	—	—	—	—	—
	2/ 3/48	S 2	—	—	—	—	—	—
25659 1/8/48	3/16/48	B 1	—	—	—	—	—	—
		S 1	—	—	—	—	—	—
		Y 1	—	—	—	—	—	—

*B 2 refers to brain of bird 2; S 2 to spleen of bird 2; Y 1 to yolk of bird 1, etc.; — means that no virus was recovered; and 0 means that no test was made.

References

[1]Beaudette, F. R., Bivins, J. A., Miller, B. R., Hudson, C. B., and Black, J. J.: Studies on the Diagnosis of Newcastle Disease in New Jersey. Am. J. Vet. Res., 9, (1948): 69-76.

[2]Todd, C.: On a Dilution Phenomenon Observed in the Titration of the Serum of Fowls Immunized Against the Virus of Fowl Plague. Brit. J. Exptl. Path., 9, (1928): 244-252.

Recovery of Newcastle Disease (Pneumoencephalitis) Virus from Mites, *Liponyssus Sylviarum*, after Feeding upon Newcastle-Infected Chickens

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THE PURPOSE of this study was to determine whether the mite, *Liponyssus sylviarum*, would become infected by feeding upon Newcastle disease-infected birds and whether this mite could then transmit the disease to susceptible birds. Mite transmission of a virus infection from one bird to another has been reported by Smith *et al.*[1] These investigators found that the chicken mite, *Dermanyssus gallinae*, is capable of transmitting the virus of St. Louis encephalitis to normal chickens by bite and that such chickens can serve as a source of virus for uninfected mites. The virus of Western equine encephalomyelitis has been isolated from *D. gallinae* collected in nature during an outbreak of the equine disease in the Southwest.[2] Reeves *et al.*[3] have recovered the virus of Western equine encephalomyelitis from wild bird mites, *L. sylviarum*, in California.

METHOD OF HANDLING MITES

A bird infested with mites, *L. sylviarum*, was obtained from a flock free from Newcastle disease infection. The mites were maintained on White Leghorn roosters. After mites were well established on the birds, the latter were inoculated with Newcastle disease virus (NDV) by the respiratory route. After a varying number of days, the birds were killed, placed in a clean, metal can, and the can surrounded by disinfectant to prevent the mites from wandering. After thirty-six to forty-eight hours, the mites were induced to migrate over a warmed surface onto clean, white wrapping paper.

The migrating mites were swept up with a wet, paper-wrapped applicator stick to which they adhered. They were then washed off in 15 to 20 cc. of broth. Five or six vials, each containing numerous mites, were collected. No attempt was made to count the mites, but the number was estimated to be from 1,000 to 3,000. The mites, killed by freezing, were washed twice in broth and ground with a mortar and pestle in preparation for egg inoculation.

At the time they were collected in broth for NDV isolation attempts, numerous mites were transferred on paper to susceptible roosters to determine whether the mites could transmit the infection to the susceptible birds. After twelve to twenty-one days, the birds were tested for susceptibility to NDV by inoculation or by testing for hemagglutination-inhibition (HI) antibodies.

EXPERIMENTAL PROCEDURE AND RESULTS

Trial 1.—One mite-infested rooster was inoculated intratracheally with Iowa 213 NDV in the form of undiluted egg fluid. On the third day, râles were heard, and, on the sixth day, the bird was killed. Mites were collected forty-eight hours after killing the rooster. No virus was isolated from the mites collected. Mites were also placed on 1 susceptible rooster, but, at the end of fifteen days, its serum tested negative for HI antibodies. Two control chicks also tested negative to the HI test.

Trial 2.—Four mite-infested roosters were inoculated with Iowa 228 NDV by the intratracheal route. On the fifth day 1 thin rooster died and was discarded. On the sixth day, the other 3 birds were killed. Forty-eight hours later, the mites were collected for egg inoculation and for transfer to susceptible chickens. Of two pooled samples of mites, NDV was isolated from both inoculums. Sample A material killed 4 of 8 embryos, while sample B material killed only 1 of 9 embryos inoculated. The broth used for washing the mites was negative for virus. Mites transferred to 3 susceptible roosters failed to produce infection in the birds since they were susceptible to the virus at the end of three weeks' exposure.

TABLE 1—Results of NDV Isolation from Mites, Liponyssus Sylviarum, after Feeding on Infected Birds

Trial (No.)	Mite-infested birds inoculated (No.)	Number of days after inoculation killed	NDV isolation from mites
1	1	6	Negative
2	4	6	Positive
3	3	7	Negative
4	3	4	Positive
5	3	4	Positive

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Trial 3.—Three roosters infested with mites were inoculated with Iowa 253 NDV. On the fourth day, râles were heard among the roosters. On the seventh day, the birds were killed, and mites were collected forty-eight hours later. No NDV was isolated from washed mites. Mites transferred to 3 susceptible roosters failed to induce infection after twenty days' exposure.

Trial 4.—Three roosters infested with mites were inoculated intranasally and in the conjunctival sac with Iowa 228 NDV undiluted egg fluid. On the fourth day, the birds were somewhat depressed and were killed. Mites were collected thirty-six hours later for virus isolation and for transfer to Newcastle-susceptible roosters. From washed mites, NDV was isolated, all embryos being killed by the inoculum. Both washings were negative for NDV. Mites transferred to susceptible roosters failed to induce Newcastle infection in the birds during seventeen days' exposure when the birds were still susceptible to intranasal inoculation with NDV.

TABLE 2—Results of Attempts to Transmit NDV to Susceptible Birds with Mites, Liponyssus Sylviarum, after Feeding on Newcastle-Infected Chickens

Trial (No.)	Birds exposed to collected mites (No.)	Mites bred on birds (da.)	Transmission
1	1	15	Negative
2	3	21	Negative
3	3	20	Negative
4	3	17	Negative
5	3	12	Negative

Trial 5.—Three mite-infested roosters were inoculated intranasally and in the conjunctival sac with Iowa 228 NDV. On the fourth day after inoculation, the birds were killed, and forty-eight hours later, mites were collected. From washed mites, NDV was isolated, all embryos inoculated being killed. Mites transferred to 3 susceptible roosters, however, failed to transmit the virus to the birds during an exposure period of twelve days. The results are summarized in tables 1 and 2.

DISCUSSION

Since a "viremia" is present in chickens at twenty-four through one hundred and eight hours following experimental inoculation with some strains of NDV intranasally, it would be probable that a feeding, blood-sucking ectoparasite could harbor NDV during that time. That NDV was isolated from mites collected from birds killed on the fourth day after inoculation in two different trials is evidence that the mite, *L. sylviarum,* does harbor the virus following feeding on Newcastle-infected birds during the time a viremia is present. The record of early and all embryo deaths indicates a relatively large amount of virus in mites at this time.

While virus was also isolated from mites collected from birds killed on the sixth day following inoculation, there was some indication that the amount of virus present was small in this collection of mites since, in one inoculum, only 1 of 9 embryos inoculated was killed, and, in the second inoculum, 4 of 8 embryos were killed. In the first trial, NDV was not isolated from mites collected from the bird killed on the sixth day. In the third trial, the virus was not isolated from mites collected from birds killed on the seventh day following inoculations.

It has been reported[4] that the virus of St. Louis encephalitis can persist from one generation of mites, *D. gallinae,* to another, for six months. From the observations reported here, it appears that the presence of the NDV in the mite, *L. sylviarum,* coincides with a viremia and that the virus does not persist in the mite.

Since known infected mites failed to transmit Newcastle infection to susceptible birds by bite under conditions of these experiments, it would appear that the mite, *L. sylviarum,* is not an important factor in the spread of Newcastle disease. The importance of the chicken mite, *D. gallinae,* in relation to the spread of Newcastle disease should be studied.

SUMMARY

Newcastle disease virus was isolated from washed mites, *Liponyssus sylviarum,* from chickens killed on the fourth day after inoculation in two trials, and on the sixth day in one instance. Virus was not isolated from mites collected from birds killed on the sixth day after inoculation in one trial and on the seventh day in another trial. Under conditions of the experiment, Newcastle disease-infected mites were unable to induce infection in adult roosters.

References

[1]Smith, M. G., Blattner, R. J., and Heys, F. M.: St. Louis Encephalitis—Transmission of Virus to Chickens by Infected Mites, *Dermanyssus Gallinae,* and Resulting Viremia as Source of Virus for Infection of Mites. J. Exptl. Med., *86,* (1947): 229-237.

[2]Sulkin, S. E.: Recovery of Equine Encephalomyelitis Virus (Western Type) from Chicken Mites. Science, *101,* (1945): 381-383.

[3]Reeves, W. C., Hammond, W. McD., Furman, D. P., McClure, H. E., and Brookman, B.: Recovery of Western Equine Encephalomyelitis Virus from Wild Bird Mites (*Liponyssus Sylviarum*) in Kern County, California. Science, *105,* (1947): 411-412.

[4]Smith, M. G., Blattner, R. J., and Heys, F. M.: St. Louis Encephalitis—Infection of Chicken Mites, *Dermanyssus Gallinae,* by Feeding on Chickens with Viremia; Transovarian Passage of Virus into the Second Generation. J. Exptl. Med., *84,* (1946): 1-6.

The Pathology of Experimental Sinusitis of Turkeys

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INFECTIOUS SINUSITIS, in the terminology of Dickinson and Hinshaw,[7] represents a specific disease of turkeys which is to be differentiated from nonspecific inflammation of the infraorbital sinus such as may occur in connection with avitaminosis A,[14] trauma, etc. The disease is of worldwide occurrence and economic importance[21] but its etiologic resolution has not been in sight until recently.

Sinusitis has proved transmissible by intrasinusoidal injection of crude exudate into normal turkeys,[3,9,11,13,15,16,22,24] but not of Berkefeld,[22] Elford collodion,[11] Chamberlain,[12] or Seitz E K[3] filtered exudate nor, as a rule, by contact.

With respect to possible bacterial agents, Beach and Schalm[1] reported the production of a sinusitis-like condition in turkeys with the fowl-coryza organism, *Hemophilus gallinarum*. From spontaneous cases of turkey sinusitis, Delaplane[4] isolated a Pasteurella-like organism which induced an exudative process in chickens retransmissible to turkeys.

Hinshaw[12] consistently recovered a gram-negative pleomorphic rod in pure culture, while Bryan *et al.*[3] did so in 7 of 23 trials; injection of such organisms failed to reproduce the disease, except in one instance each, in the hands of these investigators.

With respect to nonbacterial agents as the cause of respiratory diseases and/or sinusitis of turkeys, Harr[10] gave a preliminary account of a transmissible respiratory disease of young turkeys which differed from avian pneumoencephalitis in being nonpathogenic for chickens, but showed otherwise similar pathologic manifestations. In 1944, Minard and Jungherr[23] obtained, from an infected air sac in a turkey, an agent which could be propagated on the chorioallantois and in the yolk sac of developing chicken embryos. Intramuscular or intracerebral injection of the agent into chicks and poults caused severe depression and incoördination associated with a characteristic pathology. In 1948, Groupé, Winn, and Jungherr[9] obtained, from a case of turkey sinusitis, an agent which could be propagated in the yolk sac, where it produced lymphogranuloma-like bodies. Intrasinusoidal injection of this agent into turkeys caused sinusitis, whereas parenteral or intracerebral injections into chicks caused a syndrome similar to that seen in 1944.[23] Delaplane and Stuart[6] isolated a virus from a chronic bronchitis-like respiratory disease in chickens; the senior author[5] later found it to induce sinusitis and nervous disturbances, when injected into turkeys. Prier, Sutherland, and Beamer[25] observed an upper respiratory disease of turkeys which could be passaged intranasally in poults and chicks and in embryonating eggs. The agent was not filterable through Seitz E K pads, but passed through Berkefeld N filters in two of three trials. Hitchner[15] carried an agent, obtained from turkey sinusitis, through 22 passages in embryonating chicken eggs and found it capable of reproducing, in the original host, a disease which was grossly characterized by distention of sinuses, enlargement of Harder's gland, partial consolidation of lungs, and caseous air sac infection. Jerstad and Hamilton[16] studied a disease which manifested itself by sinusitis and/or lower respiratory infection and was transmissible by corresponding routes of inoculation and, occasionally, also by contact. An agent was propagated in the chorioallantoic chamber of chicken embryos and found capable of reproducing the disease in turkeys, while adult chickens, pheasants, and mice were refractory. The authors also made the interesting observation that the agent was present in eggs from naturally infected turkey breeders. Bryan *et al.*[3] in an addendum, announced the isolation in chicken embryos of an agent capable of producing turkey sinusitis.

While the cause of turkey sinusitis, according to these reports, appears to be a nonbacterial agent, as already anticipated by Hart,[11] definite formation of an etiologic concept is not possible at the present time. The relationship between turkey sinusitis and air sac infection likewise needs clarification, as both conditions have been looked upon as separate entities.[8] Comparative studies of the various agents are urgently needed and should place emphasis on outstanding criteria such as growth in extra-embryonic structures, experimental host range in various age groups and by routes of inoculation, regular filterability, ability to produce lymphogranuloma-like bodies, and basic pathology.[19]

Since the turkey agents isolated at this

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A preliminary report of this work was presented at Richmond, Va., June 6-9, 1948.[18]

laboratory from air sac and sinus, respectively, induced significant pathologic manifestations of similar character in inoculated chicks, the present account is believed to contribute to the characterization of such agents.

MATERIALS AND METHODS

In 1944[25] an air sac infection was studied in a geographically well-isolated turkey flock, in which the condition had been known to occur for several years. A 6-month-old Bronze turkey showed rattling sounds in the trachea and fibrinopurulent exudate in the abdominal air sacs. Negative bacteriologic examination and inoculation test with exudate on rabbits seemed to rule out fowl cholera. Injection of lung-air sac and of tracheal exudate suspensions into 3-week-old chicks by the upper respiratory and intramuscular route induced purulent air sac infections and, in one case also nervous symptoms, within about ten days. From the latter chick, a composite suspension of trachea, lung, and spleen was prepared immediately, stored in the frozen state, and passaged seventeen days later, intramuscularly, in 5-week-old chicks, with the result that 2 of 5 chicks showed air sac infection and splenomegaly. In the third passage in 3-week-old chicks, 3 of 5 chicks showed incoördination, stilted gait, and slow or jerky movement of the head, within ten to thirteen days postinoculation. The visceral changes were confined to splenomegaly. After two further intramuscular passages, the condition was carried through eight additional generations by brain-to-brain inoculation.

In a total of 13 passages involving 99 chicks, the two outstanding criteria of takes, namely, nervous symptoms and splenomegaly, were observed in 59 and 49 per cent, respectively. A significant feature was that both parenteral and cerebral inoculation produced the syndrome, while intraocular injection was followed by localized disease.

Intramuscular inoculation of 4-week-old turkeys with original turkey material (held at −20 C. for five months) and with chick material from the second passage induced essentially the same syndrome as in chicks. Bacteriologically sterile inoculums were seeded onto the chorioallantoic membrane of 11-day-old embryonating chicken eggs without the induction of definite lesions; the sixth passage was found to be pathogenic for chicks. Corresponding injections of the yolk sac of 7-day-old embryos killed the embryos within five to seven days and reached an infectivity titer of 10^{-3} in the twelfth passage. When filtered through Berkefeld N candles and Seitz E K pads, known infective, egg-propagated materail failed to induce disease. Antiviral-type serums for bronchitis, pneumoencephalitis (Newcastle disease), and fowlpox, respectively, failed to neutralize the infectivity of the agent when tested in chicks. A hyperimmune serum, however, prepared by repeat inoculation of a surviving experimental chick, likewise failed to neutralize the turkey agent.

It appeared that a transmissible, nonfilterable agent was obtained from air sac infection in adult turkeys; that such agent could be propagated on the chorioallantoic membrane and in the yolk sac of embryonating chicken eggs; and that, in parenterally or intracerebrally inoculated chicks and poults, it produced a syndrome characterized by incoördination and splenomegaly in approximately 50 per cent of the experimental birds. The details of the numerous transmission experiments are omitted, since the strain died out after prolonged storage, necessitated by the exigencies of the late war.

In 1948,[9] a sinus infection was studied in the University of Connecticut turkey flock with the result that a lymphogranuloma-like, nonfilterable agent, capable of reproducing the disease, was isolated by the yolk sac technique. Intrasinusoidal injections of turkeys were sometimes followed by air sac infection of either macro- or microscopically apparent intensity. Prolonged contact between injected and normal turkeys occasionally resulted in transmission. Intraperitoneal application in turkeys caused extensive air sac infection. Parenteral or intracerebral inoculation of chicks induced an experimental syndrome similar to that observed in 1944, except that splenomegaly was seen less frequently. Intraocular injection again failed to induce a systemic disease.

Routine bacteriologic examination of exudate from early experimental cases of infectious sinusitis in turkeys failed to yield significant organisms. Experimental turkeys failed to react to serum neutralization tests for pneumoencephalitis.

The pathologic material to be described was collected from various passages. The histopathologic methods employed were those routinely used at this laboratory, namely, Zenker's fixation for visceral tissues, formol-saline for nervous tissues, paraffin embedding, and staining with hematoxylin-triosin, and, in some instances, with Masson's trichrome-brilliant green, and Wolbach-Giemsa's solution. The possibility of complicating hypovitaminosis A was ruled out through checking experimental turkeys by nasal histopathology.[17]

For the sake of clarity, typical pathologic findings are discussed comparatively from the standpoint of the host and the organ, rather than that of chronology.

HISTOPATHOLOGY

The gross pathology of infectious sinu-

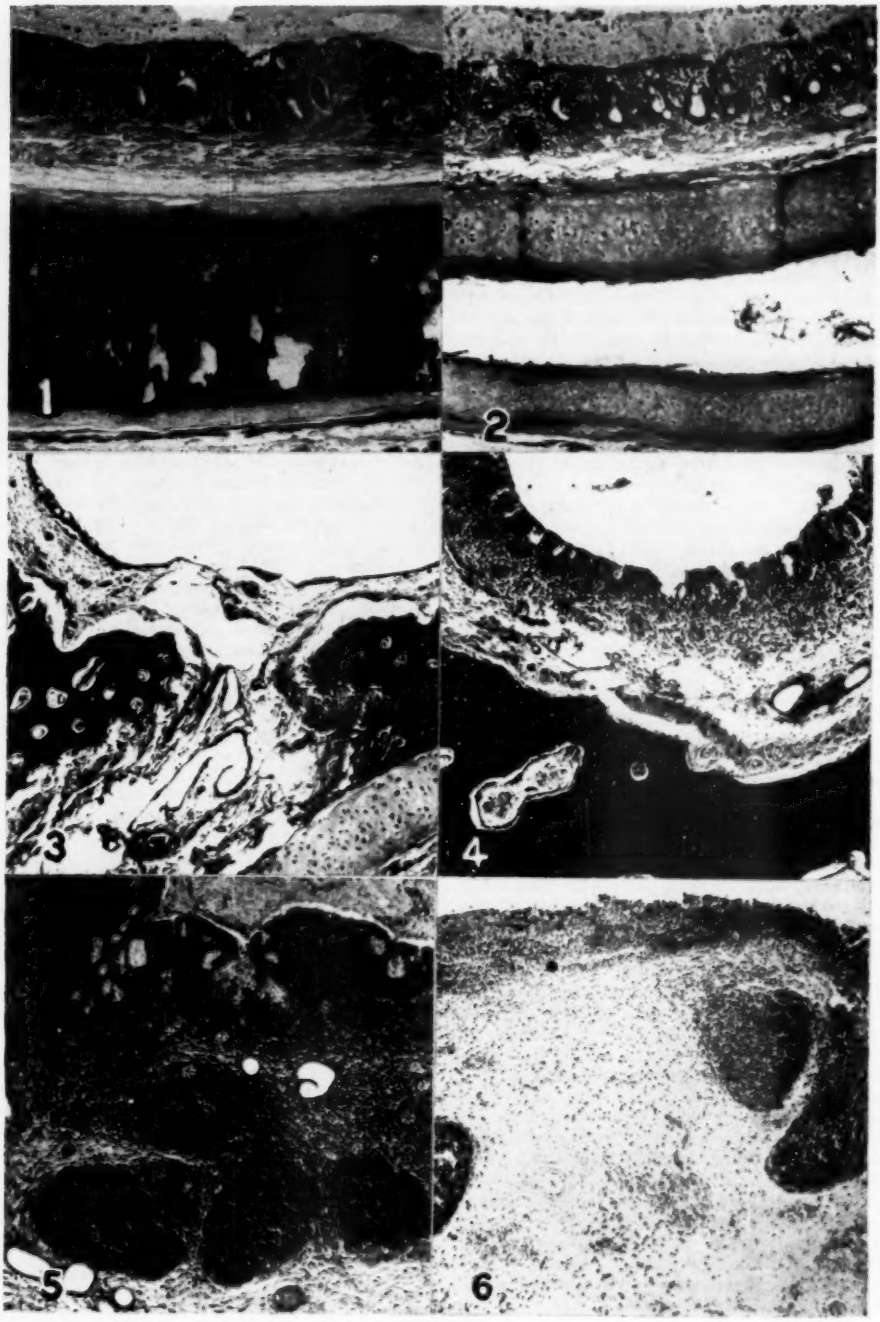

PLATE I

sitis of turkeys in spontaneous and experimental cases is well known.[11,12,22] Detailed accounts of the histopathology are lacking. In a preliminary report, Hitchner [15] stated that, in experimental cases, the epithelial tissues of affected sinuses and air sacs showed varying degrees of degeneration accompanied by proliferation of connective tissue and the lungs bronchopneumonia with an increased number of lymphoid nodules. If Sigars' observation on infectious coryza of turkeys[27]—a nonvalid term on account of its unproved etiologic relation to *H. gallinarum*—may be considered here, the sinus swellings develop primarily on the basis of "mucoid degeneration."

Trachea.—In the characterization of the group of diseases under discussion, presence or absence of clinically recognizable respiratory symptoms is often used as a criterion. It has been the observation at this laboratory that birds not infrequently fail to exhibit signs of respiratory involvement, such as rattling and coughing, and yet show definite histologic evidence of tracheitis.

The original turkeys[23] affected with sacculitis (aerocystitis) gave evidence of tracheal râles on clinical examination. Section of the trachea (fig. 1) shows marked swelling of the mucosa brought about by hypertrophy and hyperplasia of the epithelium, and by cellular infiltration, chiefly mononuclear in character, of the substantia propria. The tracheal glands are dilated and in many places filled with cellular casts. The submucous stratum is somewhat edematous and the lumen contains mucocellular exudate.

One of the original turkeys affected with sinusitis[9] failed to present audible evidence of tracheal involvement. The tracheal section (fig. 2) shows a picture almost identical with the former one, with the possible exception that the lumens of the tracheal glands are still empty, although their lining is markedly thickened as if in preparation for desquamation and consequent cast formation.

The tracheitis is essentially catarrhal and subacute in character, of a nonspecific type, and resembles, by and large, that observed in pneumoencephalitis and bronchitis of the chicken. On the other hand, it indicates involvement of the upper respiratory tract, at least at some stage of the disease, in both sinusitis and sacculitis, irrespective of clinical evidence thereof.

Infraorbital Sinus and Annexes in Adult Turkey.—Normally, the sinus is empty and lined by pseudostratified cuboidal to low columnar ciliated epithelium with occasional goblet cells. The section shown in figure 3 presents the nonaffected sinus of a turkey just coming down with the contact-transmitted disease in the other sinus. In the earliest stages, the affected sinus (fig. 4) shows flakes of mucous exudate in the lumen. The epithelium is undergoing hypertrophy and hyperplasia, assuming a cylindrical character. The goblet cells are increased in size and number, and there is loose, diffuse mononuclear infiltration in the submucosal zone, accompanied by edema and hyperemia. In the latter area, small organizing lymphofollicular structures are recognizable. The most typical picture is seen in figure 5, where the sinus from a turkey, injected two weeks previously, shows extensive mucocellular exudate in the lumen, and massive thickening of the epithelium and substantia propria, associated with dilated intraepithelial glands. The upper layer of the submucosal stratum shows dense, diffuse mononuclear infiltration and fibrosis, while the deeper zone contains massive lymphofollicular nodules.

Thus, the underlying pathologic process

Legends for Illustrations on Opposite Page

PLATE I

Fig. 1—Sacculitis. Original turkey. Trachea shows swelling of mucosa and mucocellular exudate in lumen. x 80.

Fig. 2—Sinusitis. Original turkey. Trachea shows swelling of mucosa and mucocellular exudate in lumen. x 80.

Fig. 3—Sinusitis. Turkey, contact exposure. Left infraorbital sinus is normal. x 80.

Fig. 4—Sinusitis. Turkey, same as figure 3, contact exposure. Right infraorbital sinus shows swelling of mucosa and cellular infiltration of substantia propria. Early lesion. x 80.

Fig. 5—Sinusitis. Turkey, intrasinusoidally, sacrificed fourteen days postinoculation. Infraorbital sinus shows marked swelling of mucosa and characteristic lymphofollicular hyperplasia in submucous aspect. Mucocellular exudate in lumen. x 80.

Fig. 6—Sinusitis. Turkey, intrasinusoidally. Skin in infraorbital region shows desquamation of epithelium, cellular infiltration of corium, and characteristic lymphofollicular hyperplasia in subcutis. x 80.

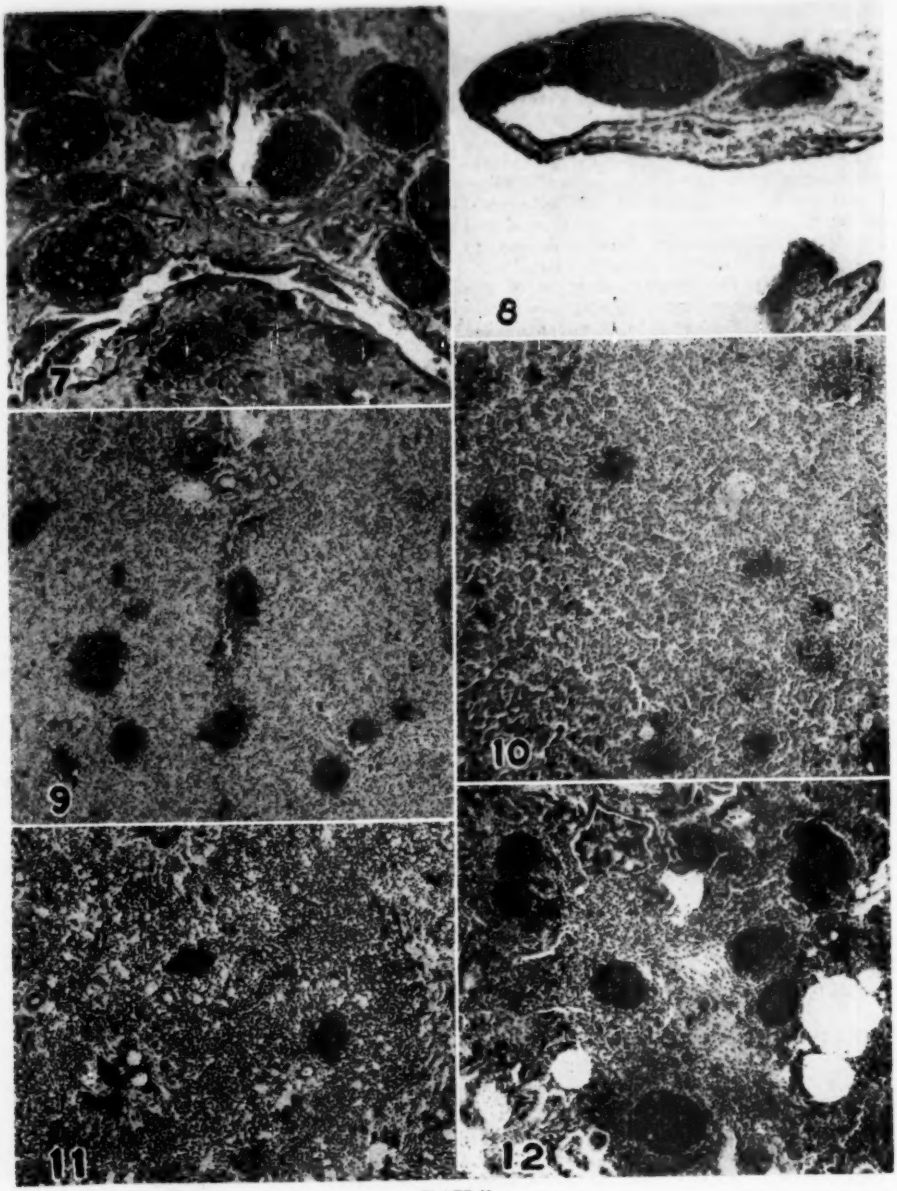

PLATE II

in the sinus seems to be one of proliferation of the epithelium, involving both its covering and secretory components, and of mobilization of the cellular defense mechanism in the submucous connective tissue. There is no evidence of necrosis or degeneration in the early stages. It can be readily seen that such thickening of the mucosa near the meatus inhibits communication with the nasal cavity and that enhanced activity of the glands results in accumulation of mucus and, thereby, the familiar fluctuating swelling in the infraorbital region.

The nasal cavity, to which the sinus stands in diverticular relation, is usually involved in the pathologic process which may be seen particularly well after unilateral intrasinusoidal injection. The respiratory mucosa, especially of the homolateral middle turbinate, is apt to show moderate swelling due to increase in size and number of the beaker cells, whereas the subepithelial aspect may exhibit numerous often evenly spaced hyperplastic lymphofollicular nodules as if the cartilage were surrounded by strings of rosary beads. The mucosa of the maxillary gland may show a pathologic response similar to that of the sinus.

The skin in the infraorbital region, following intrasinusoidal injection, is affected sometimes by gross scaling which corresponds microscopically to epithelial desquamation and leucocytic infiltration of the corium. This would not be remarkable were it not for the fact that the deeper structures again show massive lymphofollicular reaction (fig. 6).

Lung and Air Sacs of Adult Turkeys.—In about one half of the cases, intrasinusoidally injected turkeys, sacrificed three to four weeks postinoculation, showed gross and/or microscopic pneumonia. The avian lung is essentially composed of lobules, in which a tertiary bronchus in the center communicates with radially arranged alveoli. Basic pneumonic processes are either of the exudative or interstitial type or a combination of the two and may be followed in both instances by necrobiotic and granulomatous changes; they may be of lobular or lobar distribution. Bronchopneumonia, in the sense of mammalian pathology, has not been observed.

In pneumonia following turkey sinusitis, the lung may be affected by subpleural or parenchymatous foci which on microscopic examination involve several neighboring lobules (fig. 12). The alveolar walls are diffusely infiltrated by lymphocytes and present many prominent lymphofollicular nodules. Consolidation appears to be due to collapse of the alveolar spaces, while collateral emphysema predominates in the surrounding lobules.

The pulmonary and abdominal air sacs frequently exhibit extensive thickening by caseous exudate. This lesion occurs occasionally in protracted spontaneous cases and regularly follows intraperitoneal injection with virulent turkey-sinusitis agent. Histologically, the massive thickening of the air sac is due to increase of the connective tissue in the substantia propria accompanied by loose cellular infiltration and the appearance of prominent densely spaced lymphofollicular nodules (fig. 7). Both the serous parietal and the mucous visceral epithelia are implicated in the infiltrative process while the surface may be covered by seropurulent exudate. The thickened propria often shows sequesters of necrotic tissue, surrounded in part by foreign body giant cells.

Certain intrasinusoidally injected cases examined three months postinoculation appeared clinically and gross pathologically normal, except for a few millet-seed-sized yellow thickenings in the abdominal air sacs. Microscopically, however, such minute lesions duplicated the lymphofollicular

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PLATE II

Fig. 7—Sinusitis. Turkey, intraperitoneally. Grossly affected abdominal air sac shows serocellular infiltration and characteristic lymphofollicular hyperplasia.

Fig. 8—Sinusitis. Turkey, same as figure 6, intrasinusoidally, infected for ninety days. Grossly normal abdominal air sac shows three characteristic lymphofollicular foci.

Fig. 9—Sacculitis. Chick, intraperitoneally. Liver shows submiliary reticulocytic foci. x 75.

Fig. 10—Sinusitis. Chick, intracerebrally. Liver shows submiliary reticulocytic foci. x 75.

Fig. 11—Sacculitis. Poult, intracerebrally. Lung shows submiliary lymphofollicular foci. x 80.

Fig. 12—Sinusitis. Turkey, intrasinusoidally. Lung shows subpleural focus of pneumonia and characteristic lymphofollicular hyperplasia. x 80.

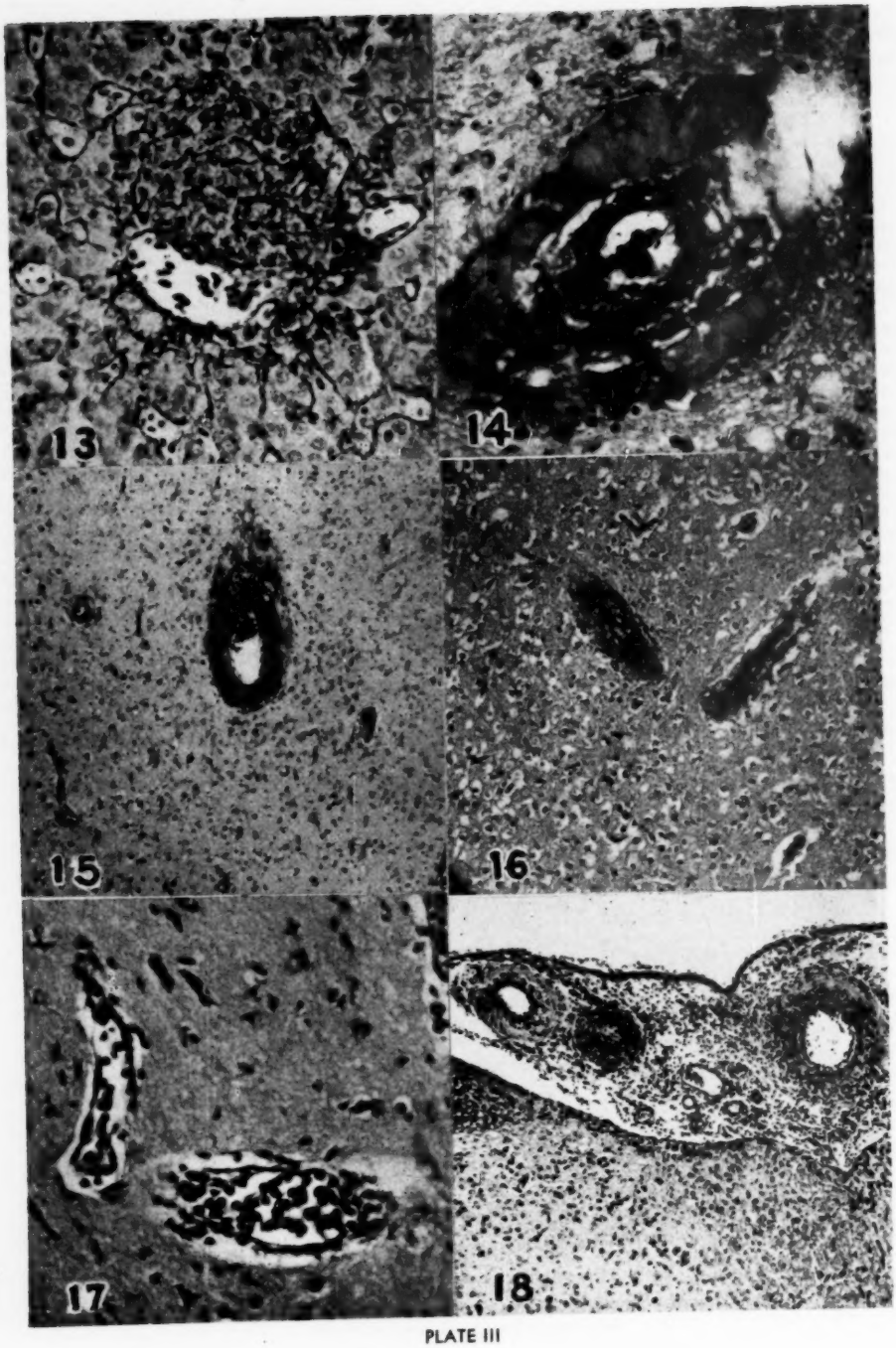

PLATE III

hyperplasia (fig. 8) of the more acute cases.

The findings in the lung and air sacs suggest that clinical sinusitis may be associated with both upper and lower respiratory tract involvement and that characteristic lesions may persist long after apparent clinical recovery.

Visceral Organs of Chicks and Poults.—As stated, the main gross pathologic criterion of successful transmission of either agent was splenomegaly and even this, on the average, in less than half of the cases. Splenic weights and gram per kilogram body weight ratios of 20 control and 20 infected chicks, 18 to 32 days old, were ascertained as 150 versus 466 mg. and 1.111 versus 3.351, respectively. Microscopically, the lesions of the spleen were represented by hypertrophy of the adenoid sheaths and variable degrees of infiltrative changes in the pulp, consisting either of noncircumscribed, or more characteristically, circumscribed, mononuclear cell aggregates. The microscopic changes were less striking than the gross alterations, but on the whole, fell in o the general pattern of the other visceral changes.

The liver in all instances appeared grossly normal. By contrast, microscopic changes were found in all birds manifesting definite takes and in about one half of the chicks, which failed to show other evidence of successful transmission. The lesions varied widely in number from submiliary (fig. 9) to localized occurrence, but were of similar cytologic character and of focal distribution without definite position in the lobular architecture, except for relation to vascular structures.

Typical hepatic changes consist of small granulomas composed of large cells with basophilic granulated cytoplasm and a large vesicular nucleus having a well-demarcated nuclear membrane and a polymorph-hyperchromatic nucleolus. Mitotic figures are common. Small groups of pathologic cells are often seen to project into, or to abut on, the sinusoids and are considered to represent the incipient stages of the granulomatous process. Even scattered pathologic cells can be recognized by their intrasinusoidal position and by their tinctorial and cytoarchitectural qualities. In Foot's silver carbonate stain, well-developed foci are seen to be permeated by a dense network of reticular fibers which normally outline only the sinusoids (fig. 13). Thus, the type cell of the granulomas appears to be an immature reticular cell.

The hepatic foci are readily differentiable from nonspecific lymphocytic aggregates which occur under normal and pathologic conditions. Affected livers also exhibit areas of necrobiosis and attempted regeneration in the form of proliferated bile ducts.

Livers of chicks injected with the sinusitis agent (fig. 10) showed essentially the same pathologic response, while the livers of adult turkeys remained normal.

In the writer's experience, the experimental lesions had no counterpart in ornithopathology but bore a striking resemblance to the reticuloendothelial granulomas described and illustrated in human pseudotuberculosis by Umlauft.[28]

The myocardium and the kidney occasionally present cellular foci similar to the hepatic changes, but somewhat less compact. Arteries near the foci may show hyaline necrosis of the media, similar to the alterations in the brain.

The lung in both chicks and poults (fig. 11) injected with the sacculitis agent are

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PLATE III

Fig. 13—Sacculitis. Chick, same as figure 9, intraperitoneally. Liver shows reticulocytic focus above central vein. Dense black-staining reticular fibers, as compared with perisinusoidal reticular fibers of surrounding normal liver tissue. Foot's reticulum stain. x 450.

Fig. 14—Sacculitis. Chick, intraperitoneally. Artery in brain shows thickening of endothelium and intima and marked hyaline changes in media. x 300. Unstained area in right upper quadrant is an artifact.

Fig. 15—Sacculitis. Chick, intraperitoneally. Artery in center and several small vessels in brain, showing inflammatory changes in wall and slight perivascular infiltration. x 80.

Fig. 16—Sinusitis. Chick, intracerebrally, sacrificed eight days postinoculation. Brain. Two arteries and several small vessels show inflammatory changes. x 100.

Fig. 17—Sacculitis. Chick, intraperitoneally. Brain shows capillaries with swollen endothelial cells and slight vascular infiltration. x 350.

Fig. 18—Sacculitis. Chick, intraperitoneally. Brain shows cellular infiltration of the subarachnoid space and of the arteries. Right artery infiltrated, middle one thrombosed, and left artery practically normal. x 80.

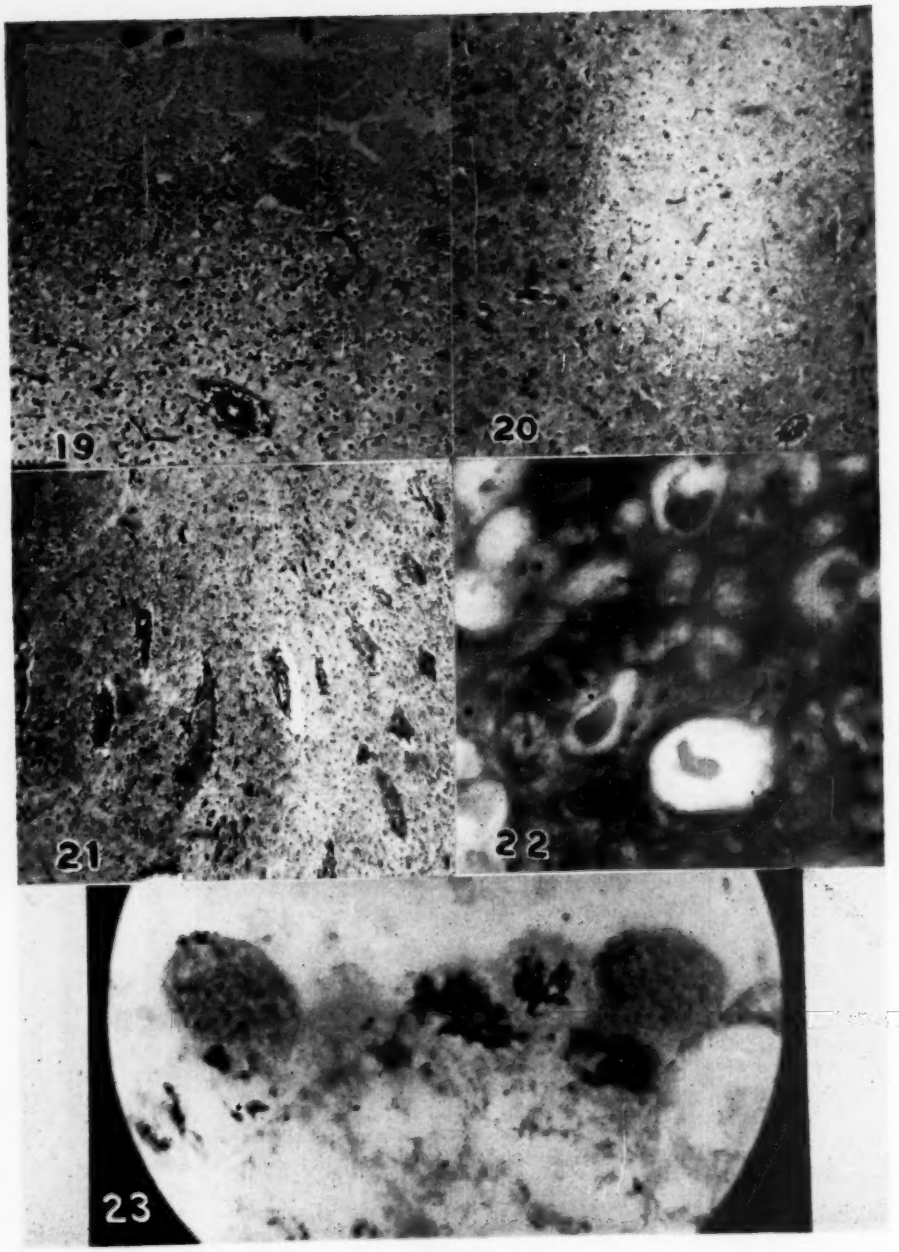

PLATE IV

sometimes affected by infiltrations indistinguishable from the characteristic lesions in the liver.

Brain of Chicks and Poults.—Experimental birds showing nervous symptoms exhibited lesions in the brain or its envelopes with regularity but with variable intensity and distribution. Characteristic vascular alterations, with or without secondary encephalomalacia, have been found in the medulla, cerebellum, mesencephalon, diencephalon, and basal ganglia, and, in rare instances, in the spinal cord. There was no consistent evidence of preferential localization, although the periventricular areas seemed to be involved most frequently As in the visceral organs, neuropathologic changes produced by the sacculitis and sinusitis agents were essentially indistinguishable (fig. 15 and 16, 19 and 20) from one another but differed significantly from central nervous system affections as seen in avian encephalomyelitis, pneumoencephalitis,[20] neural lymphomatosis, and nutritional encephalomalacia.

The basic process involves the arterioles and medium-sized muscular arteries and consists of thickening of the vessels (fig. 21), due to hypertrophy and hyperplasia of the endothelial lining (fig. 14 and 17), and necrosis and hyaline degeneration of the media (fig. 14 and 15), accompanied by a few vascular and perivascular infiltrating cells. Thereby, the process differs materially from the usual perivascular cuffs and represents essentially an endarteritis. Foci of rarefaction of the matrix or of outright encephalomalacia (fig. 19 and 20) near affected arteries are not uncommon and develop presumably on the basis of vascular occlusion, suggestive of the pathogenesis of infarcts.

The leptomeninges may show similar inflammatory changes in the arterial walls, leading occasionally to thrombosis (fig. 18), while the subarachnoid space is more prone to cellular infiltration than the correspondingly affected brain substance.

Eye of Chicks.—Inoculaton of egg-propagated sacculitis and sinusitis agent into the anterior chamber of the chick's eye caused ophthalmitis within ten to twelve days. The affected eye showed photophobia, moderate serous discharge from the conjunctival sac, clouding of the cornea, and occasionally hypopyon.

Microscopically, the condition is characterized by extensive cellulo-infiltrative changes of the cornea, iris, and ciliary body, alterations which seem to terminate abruptly at the ora serrata.

Extraembryonic Membranes of Turkey Sinusitis.—Although seeding of the turkey sinusitis agent on the chorioallantoic membrane or in its chamber failed to produce consistent gross changes, microscopic examination of the membrane showed thickening primarily due to hyperplasia of the entoderm.

The entodermal cells bordering the amnionic cavity often show acidophilic cytoplasmic inclusions within an unstained vacuole (fig. 22). Occasionally, such inclusions contain basophilic nuclear fragments or ghost nuclei. Detailed search reveals all kinds of transition figures from extravasated erythrocytes to cytoplasmic inclusions. In Wolbach-Giemsa preparation, these inclusions take an orange stain like the cytoplasm of erythrocytes and unlike the carmine red tint of true inclusion bodies. Similar structures are seen in other nonbacterial infections of the entoderm and are interpreted as pseudoinclusion bodies, probably developing on the basis of erythrophagia.

The yolk sac, following injection with the turkey sinusitis agent of a titer near 10^{-8}, shows clusters of minute coccoidal or delicate rod-shaped bodies which take a basophilic purplish stain in Giemsa preparations (fig. 23). In histologic sections, these

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PLATE IV

Fig. 19—Chick, same as figure 18, intraperitoneally. Brain shows a vascular infiltrate in lower half and encephalomalacia in upper half. x 75.

Fig. 20—Sinusitis. Chick intraperitoneally, sacrificed eleven days postinoculation. Brain shows small vascular foci in periphery and V-shaped focus of encephalomalacia (light area) in center. x 80.

Fig. 21—Sinusitis. Chick, intraperitoneally. Brain shows numerous affected arterioles. x 80.

Fig. 22—Sinusitis. Egg, into allantoic sac. Entoderm is hyperplastic and shows two eosinophilic cytoplasmic inclusions in low center believed to be remnants of erythrocytes (erythrophagia). This is a nonspecific change which may be seen in other diseases. Wolbach-Giemsa stain. x 1500.

Fig. 23—Sinusitis. Egg, into yolk sac. Yolk sac shows several clusters of lymphogranuloma-like bodies in center and a projecting cyst at either end. Wolbach-Giemsa stain. x 1200.

bodies appear primarily within the epithelial cells of the surface and can be demonstrated there with slightly greater facility than in impression smears stained according to Macchiavello. Large clumps of such "elementary" bodies not infrequently appear to be inclosed in cystlike structures or capsules which occur either free or attached (fig. 23). The characteristic bodies could not be seen in the early low titer passages with the sinusitis agent nor with the sacculitis agent, which had a titer of 10^{-3}. The livers of chicks injected with sacculitis agent sometimes show macrophages loaded with purplish coccoidal bodies which appear significant, in view of the later findings. On the whole, the structures exhibit distinct morphologic resemblance to the lymphogranuloma bodies which are depicted by Rake and Jones[26] and which are now placed into the genus Miyagawanella in Bergey's manual.[2] Specific classification of the sinusitis agent must await further studies.

SUMMARY

An account is given of the experimental pathology of two transmissible agents isolated from cases of sacculitis (Minard and Jungherr, 1944) and sinusitis (Groupé, Winn, and Jungherr, 1948) in turkeys, respectively. Both strains were nonfilterable, could be propagated in the extraembryonic membranes of the developing chicken egg, particularly the yolk sac, and produced (in about ten days) incoördination and splenomegaly in parenterally or intracerebrally inoculated chicks.

Lymphogranuloma-like elementary bodies and cysts were demonstrated in sections of yolk sacs infected with high titer sinusitis agent but not with low titer sacculitis agent.

The sinusitis agent, when inoculated intrasinusoidally into adult turkeys, produced sinusitis, followed, in some instances, by either macro- or microscopic evidence of pneumonia and air sac infection; when inoculated intraperitoneally, it produced severe air sac infection.

Histopathologically, experimental sinus infection was characterized in the early stages by severe catarrhal inflammation of the mucosa, in the late stages also by infiltration, fibrosis, and hyperplastic lymphofollicular nodules in the submucosa. Adjacent structures, such as respiratory mucosa of the nasal cavity and skin, showed similar involvement. Pneumonia and air sac infections were accompanied by development of massive lymphofollicular nodules which thereby assumed a pathognomonic significance.

Parenteral or intracerebral infection of chicks with the sacculitis and sinusitis agents produced the same highly characteristic micropathology. The liver, and to much less extent, the spleen, lung, kidney, and heart, showed multiple granulomas composed of immature reticular cells. The brain and the meninges exhibited primarily inflammation, thrombosis, and medial necrosis of the small arteries and arterioles accompanied by infarct-like areas of rarefaction or encephalomalacia. Intraocular infection resulted in anterior ophthalmitis.

On the whole, the lesions showed a general relationship to vascular structures and resembled those caused by certain members of the order Rickettsiales.

Similarity of the turkey sacculitis and sinusitis agents in cultural characters in embryonating eggs and in experimental pathology in chicks suggests an etiologic relationship or identity between the respective diseases.

The highly characteristic micropathology in chicks and turkeys constitutes an integral part of the characterization of these or similar agents isolated from respiratory diseases of turkeys.

References

[1]Beach, J. R., and Schalm, O. W.: Studies of the Clinical Manifestations and Transmissibility of Infectious Coryza of Chickens. Poult. Sci., *15*, (1936): 466-472.

[2]Breed, R. S., Murray, E. G. D., and Hitchens, A. P.: Bergey's Manual of Determinative Bacteriology. The Williams and Wilkins Co., Baltimore, Md., (1948):1115.

[3]Bryan, H. S., Prier, J. E., and Grace, O. D.: Infectious Sinusitis of Turkeys in Illinois. Vet. Med., *44*, (1949):31-33.

[4]Delaplane, J. P.: A Pasteurella or Pasteurella-like Organism as the Cause of an Infectious Sinusitis of Turkeys. Poult. Sci., *23*, (1944):247-249.

[5]Delaplane, J. P.: Some Recent Observations of Lesions in Chick Embryo Induced by the Virus of Chronic Respiratory Disease of Chickens. Cornell Vet., *38*, (1948):192-195.

[6]Delaplane, J. P., and Stuart, H. O.: The Propagation of a Virus in Embryonated Chicken Eggs Causing a Chronic Respiratory Disease of Chickens. Am. J. Vet. Res., *4*, (1943):325-332.

[7]Dickinson, E. M., and Hinshaw, W. R.: Treatment of Infectious Sinusitis of Turkeys with Argyrol and Silver Nitrate. J.A.V.M.A., *93*, (1938): 151-156.

[8]Fenstermacher, R., Pomeroy, B. S., and Malmquist, W. A.: Newcastle Disease in Minnesota. Proc. 50th Ann. Meeting U. S. Livestock Sanitary A., (1946):151-157.

[9]Groupé, V., Winn, J. D., and Jungherr, E.: Isolation of an Agent in Chicken Embryo Causing Infectious Sinusitis of Turkeys. Proc. Soc. Exper. Biol. and Med., *67*, (1948):397-398.

[10]Harr, J. F.: A Respiratory Disease of Young Turkeys. Bull. State Dept. Agric. Calif., *32*, (1943): 77-82.

[11]Hart, L.: Sinusitis in Turkeys. Austral. Vet. J., *16*, (1940):163-168.

[12]Hinshaw, W. R.: Infectious Sinusitis. In Biester, H. E., and Schwarte, L. H.: Diseases of Poultry. The Iowa State College Press, Ames, Iowa, (1948):1048-1052.

[13]Hinshaw, W. R., and Bonestell, A.: Unpublished data, 1940. Quoted in Biester, H. E., and Schwarte, L. H.: Diseases of Poultry, The Iowa State College Press, Ames, Iowa, (1948):1048.

[14]Hinshaw, W. R., and Lloyd, W. E.: Vitamin A Deficiency in Turkeys. Hilgardia, *8*, (1943):281-304.

[15]Hitchner, S. B.: The Pathology of Infectious Sinusitis of Turkeys (Abstr.). Poult. Sci., *27*, (1948):667-668.

[16]Jerstad, A. C., and Hamilton, C. M.: The Etiology of Infectious Sinusitis of Turkeys. Poult. Sci., *27*, (1948):802-812.

[17]Jungherr, E.: Nasal Histopathology and Liver Storage in Subtotal Vitamin A Deficiency of Chickens. Storrs (Conn.) Agric. Exper. Sta., Bull. 250, 1943.

[18]Jungherr, E.: The Pathology of Turkey Sinusitis. Proc. 20th Ann. Conference Lab. Work. Pullorum Dis. Control. Mimeog. Rep., Virginia Dept. Agric., Richmond, Va., 1948.

[19]Jungherr, E.: Report of the Committee on Transmissible Diseases of Poultry. Proc. 52nd Ann. Meeting U. S. Livestock Sanitary A., 1948 (in press).

[20]Jungherr, E., and Minard, E. L.: The Pathology of Experimental Pneumoencephalitis. Am. J. Vet. Res., *5*, (1944):125-134.

[21]Lee, C. D.: Sinusitis of Turkeys. North Am. Vet., *23*, (1942):715-717.

[22]Madsen, D. E.: Sinusitis of Turkeys. Utah Agric. Exper. Sta., Bull. 280, 1938.

[23]Minard, E. L., and Jungherr, E.: An Apparently New Respiratory Disease of Turkeys. Proc. 16th Ann. Conference Lab. Work. Pullorum Dis. Control. Mimeog. Rep., College of Agric., Storrs, Conn., 1944.

[24]Noordsy, J. L.: Infectious Sinusitis of Turkeys. Vet. Med., *42*, (1947):33.

[25]Prier, J. E., Sutherland, A. K., and Beamer, P. D.: Preliminary Studies on a Respiratory Disease of Turkeys. Cornell Vet., *38*, (1948):208-213.

[26]Rake, G., and Jones, H. P.: Studies on Lymphogranuloma Venereum. I. Development of the Agent in the Yolk Sac of the Chicken Embryo. J. Expti. Med., *75*, (1942):323-338.

[27]Sigars, G. O.: Infectious Coryza of Turkeys (Sinusitis or Roup). J.A.V.M.A., *114*, (1949): 16-19.

[28]Umlauft, W.: Pseudotuberkulose und Hämochromatose. Virchows Arch. f. path. Anat., *280*, (1931):18-50.

Studies on Newcastle Disease. II. The Resistance of Newcastle Disease Virus to Formaldehyde Fumigation

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THE EFFICACY of formaldehyde fumigation applied under appropriate conditions for the destruction of *Salmonella pullorum* in forced-draft egg incubators has been demonstrated by Graham and Michael[1] and Van Roekel,[2] and for the destruction of fowlpox virus by Graham and Barger.[3] The experiments reported herein were designed to determine whether fumigation procedures commonly used against *S. pullorum* would destroy Newcastle disease virus (NDV), *Tortor furens*, of chickens in incubators.

"incubation period" is the time elapsing between release of the formaldehyde and opening of the incubator, although it is realized that all of the formaldehyde is released in a few minutes.

The strain of NDV used in these studies, California 11914, was obtained from Dr. C. A. Brandly, University of Wisconsin, as lyophilized allantoamnionic fluid and was virulent for chickens and chicken embryos. The viability of the virus used in each experiment was determined by chicken-embryo inoculations.

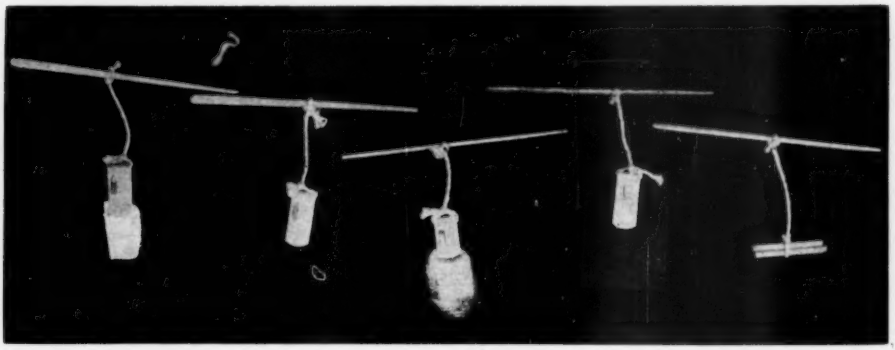

Fig. 1—Materials contaminated with Newcastle disease virus for fumigation studies.

MATERIALS AND METHODS

An electric forced-draft incubator of 31.22-cu. ft. capacity was used. The wet bulb and dry bulb thermometer readings were 85-88 F. (29.4-31.1 C.) and 98-100 F. (36.4-37.7 C.), respectively, and relative humidity 53 per cent to 68 per cent during the experimental fumigations.

Formaldehyde gas was liberated by adding 17 Gm. of potassium permanganate to 35 cc. of commercial formalin (37% formaldehyde by weight) per 100 cu. ft. of incubator space in the first series of experiments. In a second series, these amounts were doubled. The

In the first series of experiments to test the survival of the virus on different substances, the following materials (fig. 1) were contaminated by immersion in a 1 : 99 dilution of allantoamnionic fluid from chicken embryos which had died on the second or third day after inoculation with the virus:

Cotton pledge** (10 x 10 x 1 mm.)
Egg shell (10 x 5 mm.)
Glass coverslips (18 x 18 mm.)
Metal (nickel-plated steel 10 x 15 mm.)
Wood (3 pieces of white birch 2 x 15 mm. tied together with thread)

Tryptone broth (1% tryptone in 0.85% NaCl solution) was used as the diluent in all

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This work was conducted coöperatively with the Bureau of Animal Industry of the U. S. Department of Agriculture.

**The results of previous studies in incubator hygiene suggest that *S. pullorum* on cotton pledgets 1 cm. x 1 cm. x 1 mm., responded to formaldehyde fumigation in a forced-draft incubator similarly to *S. pullorum* on down of day-old chicks.

experiments. These virus-contaminated vehicles were then exposed to formaldehyde gas in the incubator.

To reduce bacterial contamination, the test materials were suspended in glass tubes, open at both ends (fig. 2). The open ends of the tubes were covered by double thicknesses of cheesecloth.

After fumigation, the virus-contaminated materials were immersed in 2 cc. of tryptone broth for ten to twenty minutes. One-tenth cubic centimeter of the suspension was then inoculated into the allantoic cavity of each of 3, or more, 10-day chicken embryos. As controls, similar test materials were allowed to stand in another incubator at 37.5 C. without fumigation for the same period and then handled similarly (table 1). The control incubator was similar to the fumigation incubator in temperature and relative humidity but without forced-draft ventilation.

In a second series of experiments, the efficacy of formaldehyde fumigation was tested against NDV in thicker films and in different vehicles. Allantoamnionic fluid, yolk, and chorioallantoic membrane were harvested aseptically from embryos dead on the second or third day after inoculation with NDV. One-half cubic centimeter each of allantoamnionic fluid and yolk were spread over approximately 20 sq. cm. in separate glass Petri dishes. A section of chorioallantoic membrane was spread over approximately 20 sq. cm. in another Petri dish. Half of a lyophilized pellet of NDV was broken into small granules in a fourth Petri dish. These dishes were then placed in the middle tray of the forced-draft incubator, uncovered, and exposed to formaldehyde gas, as described above. After fumigation, the dried material remaining in each Petri dish was resuspended in 2 cc. of tryptone broth. One-tenth cubic centimeter of this suspension was then inoculated into 3, or more, 10-day chicken embryos. A similiar set of unfumigated controls was included in each experiment.

TABLE 1—Survival of Newcastle Disease Virus in a Forced-Draft Incubator Without Fumigation

Material	Period of exposure in incubator					
	2 hr.		8 hr.		24 hr.	
	Recovery of virus	No. embryos dead/No. inoculated	Recovery of virus	No. embryos dead/No. inoculated	Recovery of virus	No. embryos dead/No. inoculated
Cotton	+	3/3	+	3/3	+	2/3
Wood	+	3/3	+	1/3	+	1/3
Egg shell	+	3/3	+	3/3	+	3/3
Metal	+	3/3	+	3/3	+	2/3
Glass	+	3/3	+	3/3	+	2/3

+ = Newcastle disease virus recovered.

TABLE 2—Resistance of Newcastle Disease Virus Exposed to Formaldehyde Fumigation for One Hour

Material	Position in tray	No. embryos dead/* No. inoculated	Results
Cotton	Bottom	7/8	+
	Middle	8/8	+
	Top	4/4	+
Wood	Bottom	1/8	+
	Middle	0/10	—
	Top	0/6	—
Metal	Bottom	0/8	—
	Middle	0/10	—
	Top	0/9	—
Glass	Bottom	2/9	+
	Middle	0/9	—
	Top	0/8	—
Egg shell	Bottom	0/8	—
	Middle	0/8	—
	Top	0/9	—

* = Newcastle disease virus recovered and identified after one-hour formaldehyde fumigation with 35 cc. of formalin per 100 cu. ft.
+ = Newcastle disease virus recovered.
— = Newcastle disease virus not recovered.

Contamination by bacteria and molds was satisfactorily controlled by treating each inoculum with penicillin (1,000 units/cc.) and streptomycin (500 μg/cc.) for thirty minutes at 37.5 C. immediately before inoculation into the embryos.

Recovery of the virus from fumigated materials and vehicles was determined by observation of lesions in dead embryos. Those not showing typical lesions of Newcastle disease were tested for the presence of NDV by hemagglutination and hemagglutination-inhibition tests. In some cases, bacterial contamination necessitated further egg passage following treatment with antibiotics, as above, to demonstrate conclusively whether NDV was present.

RESULTS

Newcastle disease virus survived at least twenty-four hours on all the materials used when exposed in the forced-draft incubator without fumigation (table 1). The incu-

bator was loaded with eggs and the fan was run, but the eggs were not turned during this experiment.

The results of a typical experimental fumigation for one hour in the first series are given in table 2. Virus on each material was exposed in the incubator in three positions, namely, the bottom, middle, and top trays. Fumigation for one hour destroyed the virus on egg shell and metal. The virus survived on wood, glass, and cotton (table 2).

(table 4) was not consistently inactivated in two- and three-hour periods when fumigated with 35 cc. of formalin per 100 cu. ft. of space. Fumigation for three hours with 70 cc. of formalin per 100 cu. ft. inactivated the virus.

DISCUSSION

In the first series of experiments, NDV survived fumigation longer on cotton than on any of the other materials on which it was exposed. Either the cotton absorbed

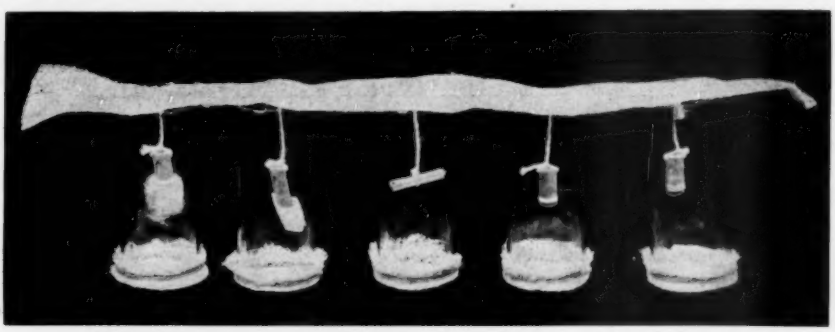

Fig. 2—Method of suspending Newcastle-contaminated materials for fumigation.

Similar experiments were conducted for three-quarters-, one-, one and one-half-, two-, and three-hour periods of fumigation. When the minimum time required to render the virus nonviable on each material was established, a second fumigation was run for this period as a check. The results are summarized in table 3.

In the second series of experiments, NDV in allantoamnionic fluid, yolk, chorioallantoic membrane, and lyophilized pellets

more virus or the penetration of formaldehyde into the pledget was less effective. Likewise, in the second series of experiments, a higher concentration of formaldehyde was necessary to destroy the virus in thick films of yolk, allantoamnionic fluid, chorioallantoic membrane, or in lyophilized pellets.

For the destruction of *S. pullorum* in incubators, a three-hour period of formaldehyde fumigation repeated three times at

TABLE 3—Resistance of Newcastle Disease Virus when Exposed to Formaldehyde Fumigation for Three-Fourths to Three Hours

Duration of formaldehyde* fumigation (hr.)	Cotton			Wood			Egg shell			Glass			Metal		
	B	M	T	B	M	T	B	M	T	B	M	T	B	M	T
¾		0			0			0			0		+	+	+
1	+	+	+	+	—	—	—	—	—	+	—	—	—	—	—
													—	—	—
1½	+	+	+	—	—	—	—	+	—	—	—	—	—	—	—
				—	—	—				—	—	—			
2	—	+	—		0		—	—	—	—	—	—		0	
2	—	—	—		0		—	—	—		0				
2	—	—	—												
3	—	—	—		0			0			0			0	
3	—	—	—												

*35 cc. of formalin per 100 cu. ft.
B = bottom tray of incubator; M = middle tray of incubator; T = top tray of incubator.
0 = not tested; + = virus recovered; — = virus not recovered.

twelve-hour intervals has been recommended. Under the conditions of the present studies, NDV was inactivated by a single fumigation for three hours, using 70 cc. of formalin per 100 cu. ft. of incubator space. In commercial incubators, the thickness of

TABLE 4—Resistance of Newcastle Disease Virus when Exposed to Formaldehyde Fumigation for Two to Three Hours

Duration of fumigation (hr.)	Cubic centimeters of formalin per 100 cu. ft.	Allanto-amnionic fluid	Yolk	Chorio-allantoic membrane	Lyophilized virus
2	35	+	+	+	—
3	35	—	—	—	+
3	35	—	—	—	—
3	70	—	—	—	—
3	70	—	—	—	—

+ = Newcastle disease virus recovered.
— = Newcastle disease virus not recovered.

deposits of dried embryonic fluids and membranes varies within wide limits. Some may be much thicker than the simulated deposits used in the present study. In such cases, conclusions drawn from this study would not necessarily be valid. To prevent thick deposits from protecting the virus, all contaminated surfaces should be cleaned thoroughly before fumigation.

SUMMARY

Newcastle disease virus (NDV) on different materials and vehicles was exposed to formaldehyde fumigation at the rate of 35 cc. and 70 cc. of formalin (37% formaldehyde gas by weight) per 100 cu. ft. of space for varying periods of time in a forced-draft incubator with the following results.

1) NDV survived two hours fumigation (with 35 cc. of formalin per 100 cu. ft.) on cotton pledgets, one hour on glass and wood, one and one-half hours on egg shell, and forty-five minutes on a metal surface.

2) In thick films of yolk, allantoamnionic fluid, chorioallantoic membrane, and lyophilized pellets, NDV survived a two- and three-hour fumigation period (with 35 cc. of formalin per 100 cu. ft. of space) but was destroyed by 70 cc. of formalin per 100 cu. ft. of space.

3) Incubator fumigation procedures recommended for the destruction of *Salmonella pullorum* appear effective in destroying NDV.

References

[1]Graham, Robert, and Michael, V. M.: Incubator Hygiene in the Control of Pullorum Disease. University of Illinois College of Agriculture and Agricultural Experiment Station. Circ. 403, 1939.

[2]Van Roekel, H.: Pullorum Disease in "Diseases of Poultry." Edited by H. E. Biester and L. Devries, Iowa State College Press, Ames, Iowa, 1943.

[3]Graham, Robert, and Barger, E. H.: Studies on Incubator Hygiene. IV. A Note on the Virucidal Effect of Formaldehyde on Fowl Pox Virus. Poult. Sci., *15*, (1936):48-52.

Salmonella Infections in Eggs of Ducks

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SALMONELLA INFECTION in ducks was first described by Manninger[9] in 1918. Since that time, several papers have been written on this subject, and may be divided under two general classifications, one dealing with food poisoning resulting from human consumption of duck eggs, the other with epizoötiologic examinations and methods of eradicating the disease from infected flocks. No definite solution has been found to the latter problem.

How Egg Infection Occurs

All authors agree that Salmonella can be transmitted by the duck, through the egg, to the duckling. In other respects, opinions are divided. Primary yolk infection in 3 to 16 per cent of the eggs laid by infected ducks was reported by Schaaf,[10, 11] Wesselmann,[14] and Schönberg.[13] Wilson,[16] on the other hand, reported no primary yolk infection but *Salmonella thompson* contamination via the shell from feces of infected ducks which had survived a duckling infection. Salmonella was not isolated from 2,000 eggs examined by Gordon and Garside,[4] although more than 50 per cent of the ovaries of the ducks laying these eggs yielded the organism upon postmortem culture. Several authors[3, 15] have examined eggs from chickens and turkeys and have concluded that, when the shells are contaminated with infected feces, Salmonella may penetrate into the egg during incubation. In Denmark, only *Salmonella typhimurium* and *Salmonella enteritidis* var. *essen* have been isolated from ducks and duck eggs.

The several possibilities of egg contamination may be itemized and then discussed. They are: (1) primary ovarian yolk infection; (2) infection of yolk during passage through the oviduct; (3) infection of shell and albumen in the oviduct; (4) infection of shell during laying; and (5) contamination of shell after laying.

1) In favor of the first item, *Salmonella pullorum* and *Salmonella gallinarum* are frequently recovered from altered follicles of infected chickens. Several authors have recorded similar opinions with regard to eggs of ducks.

We believe that culture of Salmonella from the yolks is not absolute proof of primary yolk infection, since only the shell is sterilized and a pipette is then passed through the shell membrane and the albumen to reach the yolk. We sterilized the yolk membrane itself in all of our experiments.

On the other hand, altered follicles seldom proceed naturally into the oviduct, but are found in great numbers in various positions of the peritoneal cavity.[12] Although this possibility is not always accepted,[4] we can report that we have never found an altered follicle in a duck egg.

2) Salmonella may be isolated frequently from the oviduct of the infected duck, in fact, one report[13] isolated the bacteria from 19 of 76 ducks (25%). Isolations can be made more frequently from the oviducts of laying ducks than from nonlayers, thus adding to the possibility of infection during passage through the oviduct. However, one report[12] stresses failure to demonstrate Salmonella in the yolks of four eggs laid by 4 ducks known to have infected oviducts.

3, 4) The discussion under *2* applies here in equal degree.

5) Contaminated places (nests, pens, incubators, etc.) offer ample opportunity for contact infection. Penetration into the egg has been reported by several authors, notably Lerche[8] who contaminated the surfaces of eggs with feces containing Salmonella, kept the eggs at room temperature (18 C.) for a fortnight, and then isolated the bacteria from the yolk.

Eradication Procedures

An infallible method of detecting infected ducks remains to be found. Even repeated serologic examination (Widal's test) has failed to locate all carriers. Failure to eradicate the disease from a flock of 21,000 ducks by means of blood tests has been reported.[4] Wide fluctuations in titers are reported, and these appear synchronously with functional activity of the ovary. Clarenburg[1] reports isolation of Salmonella from 1.7 per cent of 600 eggs from ducks with negative blood reactions. We conclude that even the slightest reaction to the blood test must be considered evidence of infection in practical application of the test. One must reserve judgment regarding results of tests of feces, since discharge from the oviduct may be reflected in isolation of Salmonella from the feces. Hence, in attempting

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to detect all infected ducks, fecal cultures may supplement the blood tests.

In this connection, we have several times found geese with negative blood reactions to be carriers of infection. Thus, a goose imported from England consistently yielded *S. typhimurium* in fecal cultures over a period of eight weeks. Laparotomy was performed, and samples were taken from several parts of the intestinal canal. None yielded Salmonella. The goose developed a hernia following the operation, and died. Several organs, including the oviduct, were infected, but no specific bacteria were isolated from the intestine.

EXPERIMENTS WITH ARTIFICIALLY INFECTED DUCKS

A flock of 7 ducks and 1 drake was purchased, upon ascertaining that Salmonella infection had never been diagnosed on the premises. For a fortnight, feces and eggs were examined regularly and no specific bacteria were found. On May 1, 1947, the drinking water of this flock was contaminated with a culture of *S. typhimurium* isolated from a duckling that had succumbed to the infection. This exposure was repeated on May 6, 12, and 22.

When the specific organism was found in the feces, each egg laid during the ensuing two months was examined bacteriologically. Half of the eggs for each day were cultured within eighteen hours. The other half were individually wrapped in paper and incubated at 37 C. for about a week. Eggs were readily selected for immediate examination or for incubation, because 4 ducks laid eggs with green shells; the others laid eggs with white shells.

Method.—Each fecal sample was taken from the cloaca with a glass rod, one end of which had been wrapped in gauze. Each rod was placed in a tube containing about 15 cc. of sodium tetrathionate broth, incubated for twenty-four hours at 37 C., and the contents spread onto brilliant green agar plates.

Each egg was placed in a sterile Petri dish, care being exercised to avoid injury to the yolk membrane. The surface of the yolk membrane was flamed, and 3 or 4 cc. of yolk substance was removed by means of a sterile glass tube inserted through the flamed area. This material was added to a tube (about 15 cc.) of sodium tetrathionate broth, and subjected to the same procedure as outlined above for the fecal examinations. The hands were carefully washed and disinfected with mercuric sublimate after each egg had been handled.

Results.—The tests showed that fecal excretion of bacteria was intermittent in each duck—there were periods with and periods without excretion of Salmonella. A peculiar feature of this experiment was that *S. typhimurium* could be demonstrated on the shells of newly laid eggs early in the experiment, but could not be isolated from the yolks of these same eggs. However, after incubation, about 30 per cent of these yolks yielded the same organism as the shell. About one month later, the percentage of shell infections decreased, and at this time Salmonella organisms were no longer isolated from the yolks of eggs incubated for the usual period. We have assumed that the albumen contains antibodies which prevent penetration of the organism.

The ducks did not lay eggs during the summer months, but additional examinations were made on 29 eggs laid during September. The agglutination activity of the egg whites was titrated in dilutions of 1 : 2 to 1 : 2,048, using the homologous strain as H-antigen. Readings were made after two hours of incubation in a water bath at 50 C. Two, 14, 6, 6, and 1 eggs reacted positively at dilutions of 1: 64, 1: 128, 1: 256, 1: 512, and 1: 1,024, respectively.

Titrations of the whites were negative for O-agglutinins when homologous O-antigen was used. Titration of the yolks was not possible because of inability to prepare a suitable suspension.

Antibody titers of blood serum and albumen were compared after the ducks had been separated. While caged, only 4 ducks laid eggs. Results of examination were:

Duck (No.)	H-titer blood serum	White of egg	O-titer blood serum	White of egg
114	128	64	+ ÷16	÷
116	128	128	+ ÷16	÷
101	512	128	+16	÷
113	1 024	1,024	+ 8	÷

Blood tests made in the middle of November, about a month after the ducks had stopped laying eggs, gave these titers:

Duck (No.)	H-titer	O-titer
101	128	16
110	32	8
111	128	16
112	64	8
113	64	8
114	32	8
116	16	8
drake	16	8

In January, 1948, the ducks laid a few eggs in which H-antibodies could still be found, but at titers of 1: 16 and 1: 8, only.

Influence of Egg Albumen on O-Agglutinins.—Because O-agglutinins were not demonstrated in whites of eggs laid by ducks with O-blood titers of 1:8 or 1:16, the effect of egg albumen on O-agglutination was studied. The

results indicated that concentrated dilutions of the white of a duck egg inhibited O-agglutination, and that the inhibitory effect diminished on storage at 3 C. for three months. Presence of O-agglutinins in the albumen was suspected.

That antibodies in the eggs are capable of preventing bacteria with homologous H-antigens from entering the yolk was demonstrated in the following experiment: Out of 38 duck eggs containing *typhimurium* antibodies, 20 were dipped in a culture of *S. typhimurium* and 18 in a culture of *S. thompson* (more than 90% second phase: 1,5.) After incubation for eight days, no *S. typhimurium* could be demonstrated in the yolks, whereas half of the eggs exposed to *S. thompson* contained *S. thompson* (first phase: k) in the yolks. Of considerable interest is the fact that also the strains of *S. thompson*, cultivated from the shells of the incubated eggs, had changed to first phase.

This change of phases in the yolks is based on presence of agglutinins in the whites, as explained by Gard[3], but no such change had been expected from shell cultures. We can only theorize that the bacteria were from the deeper layers, where contact with agglutinins was possible.

Results of titration of the whites with *S. typhimurium* and *S. thompson*, first and second phases, would seem to prove that the eggs in question did contain agglutinins against the second phase of *S. thompson*.

SUMMARY

All authors agree that Salmonella can be transferred by the duck to the ducklings, through the egg.

The various possibilities of infection are discussed.

In Denmark, only two Salmonella types have been isolated from ducks and duck eggs. These are *Salmonella typhimurium* and *Salmonella enteritidis* var. *essen*.

On examination of eggs laid by ducks artificially infected, only shell infection was found when investigation was made within eighteen hours of the laying.

It was possible to show yolk infection only when the eggs had been incubated for eight days at 37 C. About one month after the beginning of the experiment, it was impossible to show yolk infection, although the shell was still contaminated. The reason for this fact is that H-agglutinins, which hinder penetration, are found in the albumen. The correlation between the antibodies in blood serum and albumen is fairly good.

References

[1]Clarenburg, A.: Mededel. uit het Rijks Institut voor de Volksgezondheid te Utrecht, *3*, (1939): 118. Ref. N. Hathoffs Diss.

[2]Gard, S.: Das Schwärmphänomen in der Salmonella-Gruppe und seine praktische Ausnützung, Ztschr. f. Hyg. u. Infektionskr., *120*, (1938): 615-619.

[3]Gordon, R. F., and Buxton, A.: A Survey of Avian Salmonellosis in Great Britain. Vet. J., *102*, (1946): 187-206.

[4]Gordon, R. F., and Garside, J. S.: Salmonella Infections of Ducks and Ducklings. J. Comp. Path. and Therapy., *53*, (1943): 80-89; *54*, (1944): 61-76.

[5]Hansen, A. C.: Om Forekomsten af Salmonellabakterier hos Husdyr i Danmark. Maanedsskr. Dyrl., *58*, (1947): 369-389.

[6]Harhoff, N.: Gastroenteritisbaciller af Salmonellagruppen i Danmark. Diss., Copenhagen, 1948, 118.

[7]Kauffmann, F.: Die Bakteriologie der Salmonellagruppe. Munksgaard, Copenhagen, 1941.

[8]Lerche, M.: Enteritis-Bakterien i Enteneiern und Lebensmitteln, die mit Enteneiern hergestellt wurden. Tierärztl. Rundschau, *42*, (1936): 809-814.

[9]Manninger, R.: Paratyfus des Wassergeflügels. XXX. Allatorvosi Lapok, No. 21, No. 25, 1918. Ref. Berl. tierärztl. Wchnschr., *35*, (1919): 5, 98.

[10]Schaaf, J.: Untersuchungen über den Ansteckungskreislauf bei der infektiösen Enteritis der Enten. Arch. f. wissensch. prakt. Tierheilk, *67*, (1934): 224-238.

[11]Schaaf, J.: Die infektiöse Enteritis der Enten und ihre Bedeutung für die Nahrungsmittelhygiene. Tierärztl. Rundschau, *40*, (1934): 555-559.

[12]Schönberg, F.: Über die Infektion von Enteneiern mit Breslaubakterien vom Eileiter aus. Berl. tierärztl. Wchnschr., *51*, (1935): 474-476.

[13]Stenbæk, A.: Salmonellainfektioner hos Fjerkræ, specielt hos Ænder. Medlemsblad for Den danske Dyrlægeforening, *20*, (1937): 441-458, 467-486, 497-513.

[14]Wesselmann, A.: Bakteriologische Untersuchungen von Enteneiern. Diss., Berlin, 1935.

[15]Wilson, J. E.: Infected Egg Shells as a Means of Spread of Salmonellosis in Chicks and Ducklings. Vet. Rec., *57*, (1945): 411-13.

A Virus Infection of Turkeys and Chickens

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EVELETH *et al.*[1] described a disease of poults and baby chicks which is characterized by a high mortality during the first three weeks after hatching. Since one of the outstanding lesions of this disease is necrosis of the abdominal wall in the region of the navel, they designated the disease as "navel infection." Their previous findings had suggested that the disease was spread from hen to poult through the medium of the egg. Also from earlier studies, they concluded that the etiologic agent was a filterable virus.

EXPERIMENTAL

During the 1948 hatching season, a more intensive investigation of this disease was instituted. The work was divided into two main phases: (a) the sources and dissemination of the virus and (b) the course of the disease during the three-week period after hatching.

Four pens each containing 12 young turkey hens were used to produce the eggs for the experiment. The hens were confined to the house and the males were rotated weekly. Trap nests were used in order to identify each egg laid. All eggs that were produced in each three-week period from February 14 through May 15, inclusive, were incubated in a forced-draft incubator for a period of twenty-three days. On the twenty-fourth day, the eggs were candled, and all infertile eggs and those with dead germs were removed. The fertile eggs with live germs and the resulting poults were treated as follows.

First Hatch.—In the first of the four hatches, the eggs were placed in a separate incubator that had been cleaned and sterilized with cresol compounds. On the twenty-ninth day, all poults were removed from the incubator by an attendant, who was wearing clean clothes and who had had no previous contact with chickens or turkeys. The poults were picked up by their heads, the attendant being careful to touch no part of the birds' bodies, and were placed in new boxes. After each poult was removed, the attendant dipped his hand in a disinfectant solution before removing the next one. This disinfectant solution has been referred to by Bolin *et al.*[2] as formula No. 144.

The poults were placed in a thoroughly cleaned and disinfected battery in an isolation room. Ventilation was accomplished by forcing fresh air from the outside into the room. The temperature of the room was maintained throughout the experiment at 65 to 75 F. and that of each compartment in the brooder at 93 to 95 F. during the first week, with a reduction of approximately 5 F. each week.

In caring for the birds, the attendant walked through a pan of cresol disinfectant to enter the room, changed to coveralls and overshoes that were kept in the room and washed his hands in a solution of formula No. 144 before handling any equipment. The walls and floor of the room were washed every second day, primarily to control dust.

TABLE 1—Death Losses of Poults During the First Three Weeks After Hatching, by Breeding Pens and Hatches

Breeding pen (No.)	Death losses by hatches (%)				
	1	2	3	4	Average
1	57.1	54.5	7.0	56.4	52.1
2	16.7	26.4	8.9	32.3	22.9
3	61.5	55.3	64.4	43.8	57.3
4	45.0	49.0	63.1	84.6	58.5

Average of flock = 43.3 per cent.

The birds were fed on a ration which met the recommended allowances for poults as set forth by the National Research Council.[3]

One half of the poults from each hen was placed in one compartment, and the drinking water was treated with formula No. 144, concentration 1 : 10,000. The remaining one half received untreated water. The poults from each breeding pen were placed in separate decks of the battery.

Second Hatch.—The treatment of the eggs and poults in the second hatch followed the same pattern as those in the first hatch, with the exception that the fertile eggs from certain hens, whose offspring had indicated an exceptional livability in comparison to those from other breeding hens, were hatched in a separate incubator and were placed in two separate compartments in the battery.

Third Hatch.—The same procedure was followed for the eggs and poults in the third hatch as in the second.

Fourth Hatch.—The fourth hatch was treated as the second, excepting that, in

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Formula No. 144 used in this experiment was furnished by the Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

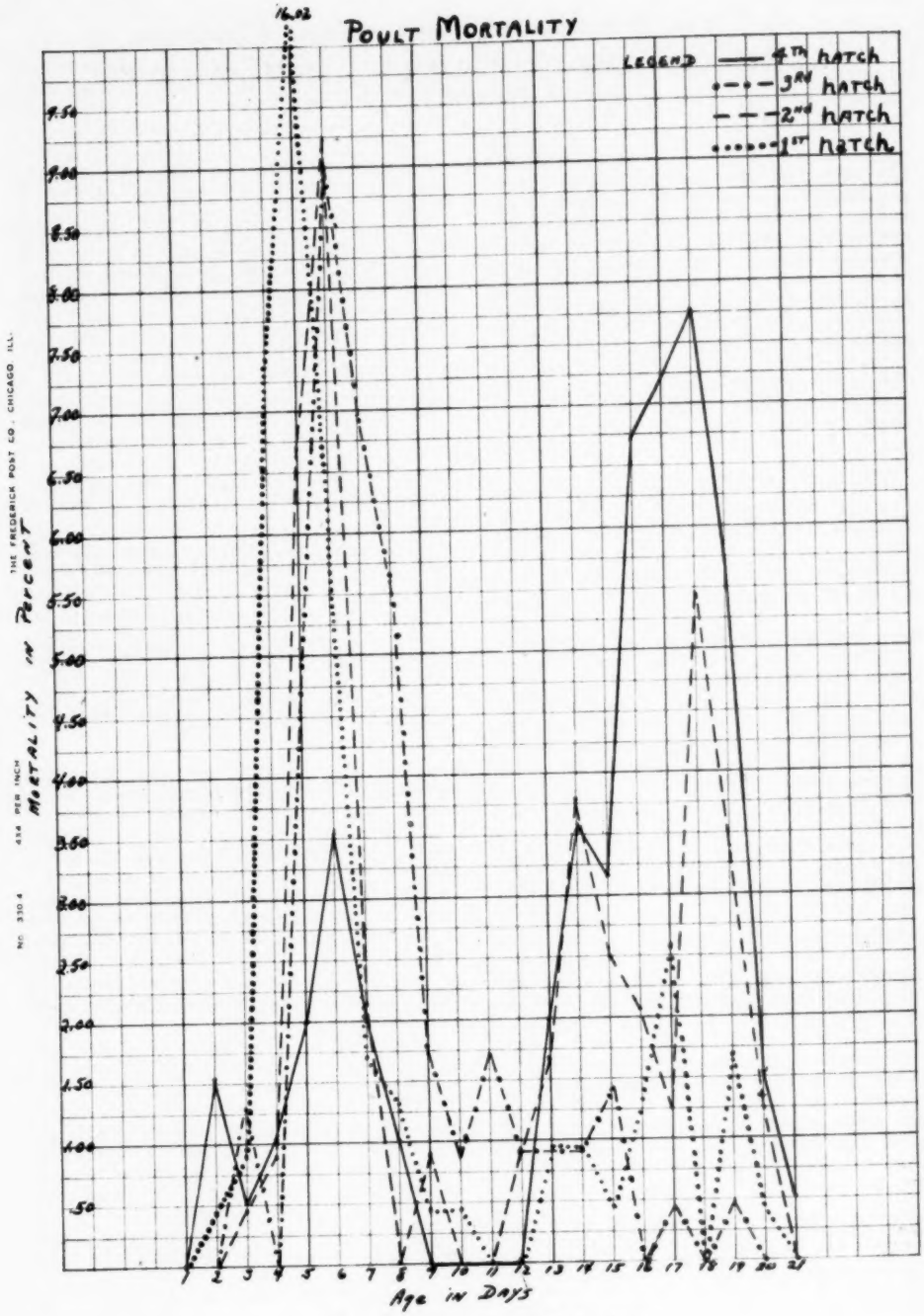

Fig. 1—Percentage of mortality by days.

alternate compartments of the brooder, the temperature was increased from 5 to 7 F. above the normal. This was done because Eveleth *et al.*[1] had found evidence that increasing the brooder temperature had been of some apparent benefit.

All poults that died, as well as some poults that died in the shell, were taken to the laboratories of the Department of Veterinary Science, North Dakota Agricultural Experiment Station, Fargo, for a postmortem examination. Gross lesions were critically examined, and attempts to isolate the virus were made. Likewise, attempts were made to isolate the virus from unincubated eggs and from ova taken from turkey hens, which had been killed for postmortem examinations.

RESULTS AND DISCUSSION

In spite of the precautions to reduce losses with extreme sanitation, the mortality was excessive. Table 1 shows the percentage loss from each breeding pen by hatches during the first three weeks after hatching. It is observed that, with the exception of those from breeding pen 2, the losses amounted to more than 50 per cent of all poults hatched. The postmortem examination showed that almost without exception those that died were affected with the virus which is being studied. The reason for the losses being lower in those poults which were hatched from the hens in pen 2 is that some of the hens consistently produced poults that were apparently free of the disease.

Figure 1 shows the percentage of mortality by days for the four hatches. Beginning with the first day after hatching, the death losses mounted rapidly, reaching a peak on the fifth or sixth day after hatching. After that, the rate of loss dropped

TABLE 2—Hatchability of Eggs from Hens with the Lowest and Highest Mortality of Offspring, by Hatches

Hen	Hatches 1 FE (No.)	1 PH (%)	2 FE (No.)	2 PH (%)	3 FE (No.)	3 PH (%)	4 FE (No.)	4 PH (%)
Low mortality								
22	4	100.0	12	83.3	10	80.0	11	81.8
24	12	100.0	10	100.0	5	80.0	16	87.5
27	11	81.8	6	83.3	13	92.3	14	64.3
29	13	92.3	4	50.0	12	83.3	6	66.7
30	13	92.3	7	71.4	9	88.9	8	62.5
High mortality								
6	5	60.0	11	45.0	8	0.0	10	60.0
36	7	57.1	6	83.3	5	80.0	12	50.0
45	12	83.3	3	100.0	11	90.9	3	100.0
53	14	71.3	11	63.6	12	58.3	9	44.4
54	10	40.0	8	50.0	12	66.7	10	30.0

FE = fertile eggs; PH = poults hatched.

TABLE 3—Livability of Poults from Hens with the Lowest and Highest Mortality of Offspring, by Hatches

Hen	Hatches 1 Poults H (No.)	1 Poults D (%)	2 Poults H (No.)	2 Poults D (%)	3 Poults H (No.)	3 Poults D (%)	4 Poults H (No.)	4 Poults D (%)
Low mortality								
22	4	0.0	10	0.0	8	0.0	9	55.6
24	12	16.7	10	0.0	4	25.0	14	35.7
27	9	22.2	5	60.0	12	0.0	9	0.0
29	12	8.3	2	0.0	10	0.0	4	25.0
30	12	0.0	5	0.0	8	12.5	5	60.0
High mortality								
6	3	100.0	5	60.0	...	...	6	83.3
36	4	100.0	5	80.0	4	75.0	6	33.3
45	10	60.0	3	33.3	10	60.0	3	100.0
53	10	60.0	7	71.4	7	85.7	4	75.0
54	4	50.0	4	25.0	8	87.5	3	100.0

H = hatched; D = died.

just as rapidly until, by the ninth or tenth day, the losses had approached the zero point. Beginning again about the twelfth day, the losses from the disease increased. When all hatches are considered, there was not the sharp peak of mortality during the last ten days as there was in the initial stage of the infection. By the twenty-first day after hatching, the death losses had again approached the zero point.

It would appear that the poults which died during the first ten days had the virus present when they were hatched, while those that died during the last ten days may have been free of the disease when hatched but contracted it from contact with the virus after hatching. This experiment tends to prove that supposition. The virus was isolated from poults which died from the disease immediately after hatching, from poults that died in the shell just prior to hatching, from unincubated eggs, and from ova taken from some of the turkey hens whose progeny showed excessive mortality and which were sacrificed for postmortem examinations. As noted above, some of the hens in breeding pen 2 consistently produced poults that were apparently free of the disease when hatched. From 7 of these hens, 201 poults were hatched for the experiment. Of these 201 poults, 36 died during the first three weeks after incubation, and 30 of those that died were lost during the last ten days of the period. One of these 7 hens was sacrificed for postmortem examination. The virus was not isolated from her. The other 6 are being held for further studies.

The mortality record of the poults from each hen revealed that, in each pen of the four hatches, treatment of the water with formula No. 144 had no effect on mortality. In only four of the 16 tests was the mortality less in the treated compartments than in the nontreated. The total losses were slightly higher in the treated than in the nontreated birds.

Some observers believe that the mortality of poults from individual hens decreases as the hatching season advances but the records do not so indicate. In fact, neither the hatchability of the eggs nor the mortality of the poults revealed any consistent trend toward change as the hatching season progressed. For a critical study of this, the 5 hens which produced poults with the highest livability of offspring and the 5 which produced offspring with the lowest livability were studied. The results are shown in tables 2, 3, and 4.

In the fourth hatch, in which the heat was increased in one half of the compartments of the brooder, the increased heat did not show any effect upon the mortality of the poults.

While this virus appears to be much more pathogenic for turkeys than for chicks, the 10- to 12-day chicken embryo is susceptible to infection. Serial transfer of the virus has been made through ten series of chicken embryos.

The disease caused by this virus has been transferred to chicks by swabbing the navels of recently hatched chicks with the amnionic fluid of embryos that had died from artificial infection with the virus. Likewise, chicks fed the virus, as well as pen contacts, have contracted the disease.

Eveleth *et al.*[1] have reported that chicks and poults, placed in brooders where pre-

TABLE 4—Hatchability of the Eggs and Livability of the Poults from Hens with the Lowest and Highest Mortality of Offspring

Hen	Hatches 1			2			3			4			Total		
	FE	PH	PD	FE	PH	PD	FE	PH	PD	FE	PH	PD	FE	PH	PD
Low mortality															
22	4	4	0	12	10	0	10	8	0	11	9	5	37	31	5
24	12	12	2	10	10	2	5	4	1	16	14	5	43	40	10
27	11	9	2	6	5	3	13	12	0	14	9	0	44	35	5
29	13	12	1	4	2	0	12	10	0	6	4	1	35	28	2
30	13	12	0	7	5	0	9	8	1	8	5	3	37	30	4
Total	53	49	5	39	32	5	49	42	2	55	41	14	196	164	26
High mortality															
6	5	3	3	11	5	3	8	0	0	10	6	5	34	14	11
36	7	4	4	6	5	4	5	4	3	12	6	2	30	19	13
45	12	10	6	3	3	1	11	10	6	3	3	3	29	26	16
53	14	10	6	11	7	5	12	7	6	9	4	3	46	28	20
54	10	4	2	8	4	1	12	8	7	10	3	3	40	19	13
Total	48	31	21	39	24	14	48	29	22	44	22	16	179	106	73

FE = fertile eggs; PH = poults hatched; PD = poults died.

viously there had been losses due to this virus, contracted the disease and died.

It appears certain that this disease is not the omphalitis or navel infection that is ordinarily associated with faulty incubation or insanitary conditions. That disease syndrome is usually the result of several bacteria of hatchery origin and thorough cleaning of the incubators and hatchery usually prevents further losses. The disease reported here is apparently a virus and is present in some of the poults before they are hatched. In fact, some breeder hens are known to carry the virus in their ovaries.

SUMMARY

1) In a series of four hatches from 48 turkey hens, data have been recorded on the death losses of the poults. These losses were caused by a virus infection.

2) The virus was isolated from poults which died from the disease, from poults that died in the shell just prior to hatching, from unincubated eggs, and from ova taken from some of the turkey hens whose offspring showed excessive mortality.

3) The disease followed a regular pattern, with the first peak of mortality on the fifth or sixth day after hatching, and a second peak on or about the sixteenth to eighteenth day. The losses approached the zero point about the twenty-first day after hatching.

4) The progenies from some hens apparently were hatched free of the virus, since they did not succumb to the disease immediately after hatching.

5) Neither treatment with formula No. 144 nor a higher-than-normal brooding temperature had any beneficial effects in preventing losses from the disease.

6) The virus appeared to be much more pathogenic for turkeys than for chicks. However, the 10- to 12-day chicken embryo was highly susceptible. Serial transfers were made through ten series of chicken embryos.

7) The disease was transferred to chicks by feeding and by swabbing the navels with amnionic fluid of embryos that had died with artificial infection. Chicks also contracted the disease by pen contacts.

8) It appears certain that the disease was not the one usually referred to as "navel infection," which is associated with faulty incubation or insanitary conditions.

References

[1]Eveleth, D. F., Bolin, F. M., and Goldsby, Alice I.: Studies on Navel Infections of Chicks and Poults. North Dakota Agric. Exper. Sta. Bimonthly Bull., 9, (1947): 160-162.

[2]Bolin, F. M., Goldsby, Alice I., and Eveleth, D. F.: Di-isobutyl Phenoxy Ethoxy Ethyl Dimethyl Benzyl Ammonium Chloride as an Aid in the Prevention of Protozoan Diseases of Poultry. Vet. Med., 42, (1947):306-310.

[3]Cravens, W. W., Almquist, H. J., Norris, L. C., Bethke, R. M., and Bird, H. R.: Recommended Nutrient Allowances for Poultry. A report by the Subcommittee on Poultry Nutrition. Nat. Res. Council, 1946.

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